

The case for an interim test-ban

Against next year's review conference of the Non-Proliferation Treaty, the nuclear powers need something tangible to offer their critics. An interim fixed-term comprehensive test-ban is the best opportunity.

Mr Boris Yeltsin's journey through the Western world last week was wrongly overshadowed by his failure to appear on the tarmac at Shannon airport to greet the prime minister of Ireland, Mr Albert Reynolds; it is significant, and more important, that Yeltsin had earlier told the General Assembly of the United Nations that the time had come to sign the Comprehensive Test-Ban Treaty (CTB). Yeltsin and his Russian predecessors are late converts to this cause. In the closing months of the Carter presidency in 1979, the Soviet Union was the one among the signatories of the existing Partial Test-Ban Treaty which demanded an unrealistically large number of monitoring sites in the United Kingdom to make sure that the British government was not renegeing on its obligations. The requirement was enough to kill an agreement that might otherwise have been in force for the past 15 years.

Yeltsin's problem is probably the inverse of that of his predecessor-but-three, Leonid Brezhnev. In 1979, with the Cold War still being fought, keeping the other side guessing was a reasonable tactic. Now, Yeltsin is probably as embarrassed by the nuclear weapons still intact on Russian territory as are Russia's neighbours, many of them parts of the old Soviet Union. It is not simply that the authorities in Germany appear, in the past few months, to have been adept at picking up people bent on smuggling Russian fissile material out of Russia, but that the physical dismantling of Russian warheads is turning out to be a more formidable task than anybody had expected. "Cannot this problem go away?", Yeltsin may have been asking himself last week.

Sadly, for him and the rest of us, there is no way of doing that without agreement. But there is no reason why the CTB, like other important steps along the road to nuclear containment in the past decade, should not be a gradual process. The way to start is with an interim agreement, say for the next five and a half years. One benefit of such an arrangement between the three signatories of the partial test-ban treaty is that it would carry substantial weight with next year's conference of the signatories of the Nuclear Non-Proliferation Treaty, all of whom will want to know what progress has been made towards nuclear disarmament. Another is that it would put cryptonuclear powers on the spot: would clandestine developers of nuclear weapons agree to forgo tests for the next five years and, afterwards, perhaps indefinitely? That would be a neat way in which the existing nuclear powers (Yeltsin's among them) could neatly turn the tables on their critics essentially by asking that they should either quit the NPT because they cannot stomach its terms or that they should put

their plans on ice for the next five years, by which time the world will be a different place. Either way, we should then know the black sheep. □

Conflicts of interest

Academic institutions must be more zealous in policing arrangements between employees and industry.

THE latest twist in the great genome sequencing saga has been predictable for several years. The circumstances are described at great length in last week's issue (*Nature* 371, 363–364 & 365–366; 1994) and on page 463 of this week's. J. Craig Venter's gene-sequencing organization, the Institute for Genome Research, in partnership with Human Genome Sciences Inc. and SmithKline Beecham, has stolen a march on the rest of the academic community by producing 150,000 genomic tags for human genes. Two aspects of the development rankle with the research community. First, the academic community feels upstaged. Second, people are reluctant to sign agreements with holders of this information (which is otherwise to be freely available) that will give the latter proprietary rights to inventions that may ensue. A meeting to be held in Washington this week cannot make the underlying problem go away.

The crucial question is the degree to which academic researchers can reasonably be involved with the commercial exploitation of discoveries arising from their work. It is not, of course, a novel question; it has become a clamant question only in recent decades, since the emergence in the 1970s of biotechnology as a means of making novel and useful products. Since then, many academic researchers have initiated the formation of commercial companies, sometimes doubling as officers of the companies or as consultants to them. Usually, the academic entrepreneurs end up owning part of the new company's equity.

Just how and where to draw the line between an academic researcher's academic and commercial interests is not an easy business. Academics' prime responsibilities are for the education of the young and the deepening of the general understanding of the natural world, but they are also (rightly) held to have a general responsibility for the improvement of industry, either through their publications or by providing direct assistance, as through consultancy.

None of that implies that they can be indifferent to the



commercial potential of discovery. So much is vividly illustrated by the occasions when people have decided not to seek patent protection for a new development, believing that an invention put into the public domain is in the public interest, only to discover that important applications of their work are then protected by commercial companies with the wit to see that a sharp patent lawyer can be as valuable as a talented researcher. The discovery of monoclonal antibodies in 1975 is a classic, but not the only, illustration.

These days, most academic researchers are more pointedly reminded of the potential proprietary value of their work. For one thing, most governments applaud the academic-entrepreneur as a harbinger of small or even large business. For another, most universities and institutes are new sources of income, looking enviously at organizations such as the Wisconsin Research Foundation as a means of turning academics' inventions into cash-flow. What institutions fail to appreciate is that these are radically different solutions to their problems, on the wrong and the right sides respectively of the line defining acceptability.

For an academic also to be an officer of a commercial company is an intolerable conflict of interest. At the least, it means that if the company gets into difficulties, the academic has a fiduciary responsibility to the company's shareholders to put their interests ahead of those of students. More seriously, such a person cannot be expected to give impartial advice to the generality of companies that might look for it. And however strong-minded he or she may be, the suspicion that academic resources are being put to commercial use cannot easily be countered. Even to be a non-executive member of a company's board can create conflicts.

The Wisconsin solution, on the other hand, is acceptable. If a university or institute is able to patent and exploit the discoveries of its members, it can share the proceeds (if any) with the academic in the full knowledge that students' interests will not be put in hazard, and that the person concerned will still be able to offer impartial advice to all and sundry. One snag is that the patent business is a jungle, often impenetrable by academic institutions. In recognition of that, the British government half a century ago set up the National Research Development Corporation (now privatized) to do just that. Several organizations have sprung up in the United States to offer just such a service.

In between, there is much fuzziness. It seems generally to be accepted among academic researchers and their institutions that paid consultancies are permissible, but even that is not crystal-clear. In reality, only fellow-academics can tell accurately whether a person's agreement with an external company compromises his or her capacity to carry out acknowledged academic duties. But curiously, as things are, most institutions can be satisfied by an academic's undertaking that a consultancy will occupy no more than some specified time each week, month or semester. That is insufficient. People's contractual arrangements with outsiders should be the common knowledge of their close colleagues.

How does this bear on the use likely to be made of Venter's gene tags? Many people will happily sign up for general access to his database. They will include those who

suspect (probably correctly) that it contains important clues to the way in which important genes are switched on and off in the course of the development of the human embryo; with appropriate software, they may be able to pick out significant patterns in the succession of gene expression that will allow them to say something important about the ontogeny of human beings. That, of course, is also an honourable goal.

On the other hand, people (if there are any) contracted to commercial companies to help find the genetic basis for schizophrenia in the hope that the company will then develop drugs more effective than the phenothiazines will be in a fix; they will not be able to sign the agreements with Venter's commercial partners now on offer with an easy conscience, or without the fear of crippling lawsuits. But that is precisely the quandary the doctrine of ownership in intellectual property creates; you cannot use other people's discoveries without charge, but then protect your own. □

Universities for export

A plan to build a British university in Thailand deserves the ridicule it will attract.

BRITAIN'S long-standing connections with Thailand (previously called Siam) appear to have flourished in a remarkable way; a consortium of British universities is planning to operate a British university there as soon as 1996 (if the Thai government gives its approval for the project). The logic behind the proposed arrangement is impeccable. Many young people from Thailand arrive in Britain every year to register for undergraduate courses of various kinds, computer science conspicuous among them. Why not save the students (and their parents) the air fares, the time and the trouble, and move the university to Thailand instead?

Inevitably, the project has won the blessing of the British government, which is a zealot for anything that smacks of adding to Britain's export business. Indeed, the trade minister, one Richard Needham, has been musing to the *Financial Times* about the potential for following suit "in (*sic*) the Pacific Rim" and in Latin America. The plan is that the new institutions should be staffed by British academics, presumably on part-time secondment from home institutions. Nothing appears to have been decided about research, perhaps because it is assumed that teachers at the new institution will have enough of that on their long furloughs every year.

This plan may be the nearest thing yet to an effective mockery of the university as an intellectual and cultural institution. What is a university without roots in the culture whose young people go there for education? What is a university if, by ignorance, it has nothing to say about how that culture conducts its business as a society? What if it has only the kinds of engagement in research that will satisfy the needs of self-interested local businesses? Even British universities' shortage of financial support, well catalogued over several years, can hardly justify such a cynical disregard of the purposes for which universities exist. □

HGS seeks exclusive option on all patents using its cDNA sequences

London. In a move that will have a wide impact on the molecular genetics community, the gene-sequencing company Human Genome Sciences Inc. (HGS) has released details of the terms that university-based scientists will have to accept if they wish to work with sequences produced by HGS's Institute of Genomic Research (TIGR).

If — as expected — the terms are endorsed by a number of major research institutions, the move will clear the way for the publication of information on almost 150,000 cDNA sequences compiled by TIGR, headed by Craig Venter, formerly of the National Institutes of Health.

But the fact that the terms provide HGS with the opportunity to retain control over the commercial applications of knowledge derived from the sequences, and in particular the discovery of any genes arising directly from this knowledge, means that controversy over the company's actions is unlikely to disappear.

The publication of the agreement follows an announcement on Monday by HGS and SmithKline Beecham, which last year took a major equity stake in HGS, that they are planning to open up shortly to the academic community a Human cDNA Database containing all cDNA sequences compiled by TIGR at its sequencing laboratory in Gaithersburg, Maryland.

It also follows last week's announcement by rival pharmaceutical company Merck Sharpe & Dohme that, in apparent protest at SmithKline's efforts to 'corner' the commercial use of the human genome, it is financing a separate sequencing project at the University of Washington, St Louis, whose results will be placed unrestricted in the public domain (see *Nature* 371, 365; 1994).

The main condition of access to the TIGR database is that the institution for which an investigator works must sign an 'option agreement' with HGS, under which HGS will have an exclusive option on any patents arising from research using the database sequences.

As part of a separate 'access agreement', an investigator must agree that all scientific papers arising from research using the TIGR sequences will be submitted to HGS at least 30 days before publication, giving the company time to decide whether a patent application should be filed. (HGS will have the right to demand a further 30 days delay to pursue a patent application.)

If it is interested in pursuing the discovery commercially, the company will have six months in which to negotiate an accept-

able deal with the university. At the end of six months, if HGS decides not to pursue the negotiations further, then the university (which will own the patent rights to the invention) will be free to enter into negotiations with other companies interested in taking out a licence on the patent.

According to George Poste, research director of SmithKline, the terms of the agreement are little different from those that any commercial sponsor would attach to its support for a university-based group of researchers. He argues that free access to the cDNA database should be considered a com-



The Institute for Genomic Research has already sequenced over 100 million nucleotides.

parable form of support for university scientists to a grant which they might receive directly from a pharmaceutical company.

But SmithKline's conditions are unlikely to please other companies, who will be required to enter into commercial agreements with HGS if they wish to use the TIGR sequences in their own development work. "Commercial organizations are welcome to approach us, and we would be happy to discuss terms for collaboration," says Poste.

SmithKline executives dismiss Merck's move to establish a separate 'public domain' sequencing operation on the grounds that HGS already has a substantial head start in compiling cDNA sequences through the efforts of TIGR. "We have always realized the importance of moving rapidly, and it seems that others are now planning to start on a process which we have almost completed," says William Haseltine, the chief executive officer of HGS.

Both Poste and Haseltine defend the relatively aggressive stance that HGS has taken in driving hard bargains with commercial competitors who would like access to the TIGR data. "The essence of industry is competition," says Poste, who has described genes as a form of "currency" to be used in negotiations with potential partners. "Whether this is expressed through actions

to boost your own competitive position, or by attempts to reduce another company's ability to compete — that has long been the cut and thrust of industry."

At the same time, the agreement could cause difficulties for academic geneticists who hold equity stakes in, or act as directors of, small gene-hunting companies. If they wish to use TIGR data, an agreement giving HGS a first option on the use of patents arising from the data could come into conflict with a legal commitment to provide their company with all information relevant to its interests.

"For academics with company ties which go beyond consultancy arrangements or research grants, and may include a legally binding obligation [to a company], it is their own ethics that will determine what they do with the data," says Poste. "But I hope that they would recognize a potential conflict of interest."

The agreement released on Monday is the latest product of prolonged effort by HGS to develop terms for access to and the use of TIGR data. Earlier versions of so-called "material transfer agreements" provoked widespread protests in the academic community, partly as a result of complaints from university patent lawyers who felt that HGS was restricting the ability of their institutions to negotiate separate deals with other commercial sponsors of research.

Overall, the dispute has provoked widespread debate over whether the generation of a 'transcript map', involving the mapping of cDNA sequences on to a physical map of the genome, should be carried out in the public or the private domain.

HGS and SmithKline officials remain unapologetic about the stance that they have taken. "We have committed more than \$100 million to Craig Venter's work at TIGR, as well as an extra \$8.5 million in making the database available to the academic community, and feel that we have a reasonable right to profit from any results that emerge from something that we have made an investment in," says Poste.

But Merck, backed by a number of other pharmaceutical companies who fear that HGS could exert excessive control over the commercial applications of cDNA sequences — and claim that this could reflect back negatively on the academic community — plans to maintain its strategy. "The more genomic information is available for researchers to use on an unrestricted basis, the more likely that we will see important discoveries and progress in our knowledge of the human genome," says Merck spokesman Jeff Sturchio.

David Dickson

Japan turns to US for help in launching gene therapy

Tokyo. Japan's dependence on the United States and other Western countries for expertise in the whole field of gene therapy has been highlighted by the process of government screening and approval of Japan's first proposed use of gene therapy.

Some Japanese researchers now fear that the application of gene therapy in Japan may give rise to renewed US criticism that the country is taking a 'free ride' on the basic science and technology of Western nations.

Last week, a newly established gene-therapy screening committee of the Ministry of Education, Science and Culture (MESC) met to discuss a proposal by a group at Hokkaido University to apply gene therapy to a 3-year-old boy suffering from adenosine deaminase (ADA) deficiency, an enzyme deficiency that can cause a breakdown in the immune system. The meeting of the MESC committee followed a meeting a week earlier by a similar committee of the Ministry of Health and Welfare.

Both committees, which have some overlapping members, decided to form a working group comprised of the seven members they have in common to examine the safety and efficacy of the proposed technique, a modification of the world's first gene-therapy treatment carried out by French Anderson and Steven Rosenberg at the US National Institutes of Health in 1990.

The retroviral vector to be used by Hokkaido University will be imported from the United States, and the working group will be entirely dependent on US data for assessing its safety.

Takehiko Oura, head of the university's hospital, told a press conference last month that the university has been in frequent contact with officials in the United States since June in order to obtain the necessary documentation to establish the safety of the technique.

At the end of August, when the university applied simultaneously to both ministries for approval of its proposed treatment, three published papers were submitted to substantiate the efficacy and safety of the technique. But this material was apparently not considered to be sufficient by the Ministry of Health and Welfare, and more documentation is now being sought.

The US Food and Drug Administration (FDA) has large amounts of data on the vector, which was developed by Gene Therapy Inc., a US company established by French Anderson. But the university has not yet been able to obtain all the FDA documentation.

The case has drawn attention to Japan's lack of an infrastructure either for developing vectors for gene therapy, or for assessing

their safety. Seven leading Japanese pharmaceutical companies, with financial support from the Ministry of Health and Welfare, are planning to set up a joint venture to develop vectors, says Mitsuru Miyata, with widely-respected editor of the newsletter *Nikkei Biotechnology*. By developing its own vectors, Japan should be able to reduce its payments of royalties to US companies.

It was originally planned that the joint venture would also assess the safety of vectors. But it has now been decided to contract out such work to the US company Microbiology Associates, which performs this role in the United States under FDA guidelines, says Miyata.

In addition, the Ministry of Health and Welfare is planning to provide a small grant of about ¥10 million (US\$100,000) to Japan's National Institute of Health to assess the safety of viral vectors.

One fundamental problem facing Japan is the lack of chimpanzee colonies to test the safety of vectors. There are only two colonies in Japan, one run by the government in Tsukuba science city and the other private. Both are preoccupied with testing the safety of treatments for AIDS and viral hepatitis, and, given the limited government funding for research in Japan, it is unlikely that this situation will be improved in the near future.

David Swinbanks

Committees split over opening up proceedings

Tokyo. Japan's two gene-therapy screening committees may serve parallel roles (see left), but they have adopted a very different attitude towards coverage of their proceedings by the Japanese media.

Television cameras and the press were allowed into the meeting two weeks ago of the screening committee of the Ministry of Health and Welfare, creating an atmosphere reminiscent of the open meetings of the Recombinant DNA Committee in the United States.

In contrast, the more conservative Ministry of Education, Science and Culture refused to let the press into its meeting.

Education ministry officials argue that, while the health and welfare ministry committee has to cover medical issues of social importance (and therefore requiring press coverage), their committee will focus more on research.

But the fact that the two committees have formed a working group on the safety and efficacy of proposed gene therapy comprised of the same seven members effectively belies any difference between the two committees. Furthermore, it is well known in Japan that government subcommittees, rather than their parent committees, are the more important decision-making bodies.

D. S.

UK charity cuts research funds

London. Britain's largest medical charity, the Imperial Cancer Research Fund (ICRF), announced last week that financial pressures have forced it to reduce overall spending on research by more than 10 per cent next year. The decision will result in the closure of several ICRF laboratories, and substantial cuts in recruitment and support for research fellows.

Sir Walter Bodmer, the director-general of the ICRF, says that the fund has decided to give a stronger focus to its support for basic research — in particular in its central laboratories in London and at Clare Hall, in Potters Bar, Hertfordshire — and on clinical research.

Research units to be closed in London include the Tumour Immunology Unit at University College and oncology groups at the Royal London Hospital and the Institute of Child Health. Funding for the ICRF's Molecular Pharmacology Unit in Dundee and its laboratory at the Institute of Molecular Medicine in Oxford will also be cut back.

Bodmer says that the work of some of the groups being closed may be continued in ICRF's main laboratories. At the same time,

about 30 of the fund's 1,600 posts may disappear.

The reduction in the ICRF's operating budget — almost 90 per cent of which is spent on research — from a planned £60.5 million (US\$95 million) to £54 million next year has been made necessary largely by a drop in income from legacies. These represent almost three-quarters of the ICRF's voluntary income, and grew by about 15 per cent a year during the 1970s and 1980s.

Anticipating (wrongly) that this trend would continue, ICRF has been operating at a deficit of several million pounds a year for the past few years. "We had hoped that increases in income would meet our additional expenditure, but there came a point at which we felt this approach was no longer viable," says Bodmer.

According to Bodmer, ICRF's financial difficulties highlight the importance of the recommendation of a recent working group on research in the National Health Service that charities be relieved of covering the basic health-care costs of patients in hospitals where they fund clinical research units (see *Nature* 371, 275; 1994).

D. D.

Congressional gridlock sinks US science bills

Washington. Legislation central to the science, technology and environmental policies of the Clinton administration is set to be lost, much of it probably for good, when the US Congress breaks up at the end of this week for November's elections.

The failure of key bills such as the National Competitiveness Act — which would authorize new technology programmes, including an ambitious research programme for the information super-highway — as well as a tranche of proposed environmental legislation, is partly the result of the Republican party's decision to use its 45 votes in the Senate to block as much as it possibly can in the run-up to November's poll.

But the poor legislative track record of the 103rd Congress is also being blamed on the ineffectual backing the administration has given to its own political agenda, as well as to a fragmented committee structure that makes it difficult for science and technology measures to pass both the House of Representatives and the Senate.

Congressman George Brown (Democrat, California), chairman of the House of Representatives Science, Space and Technology Committee, says he is "bitterly disappointed" with the progress that his committee was able to make with its legislative programme.

Science bills that have failed to advance

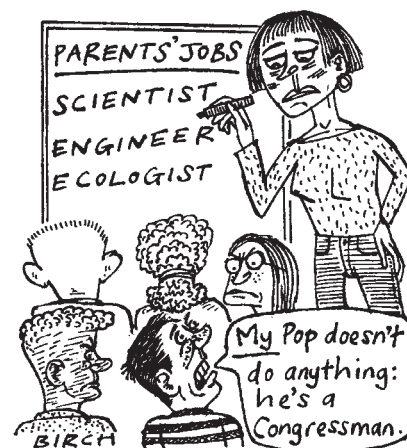
during the session include a fusion and high-energy physics act (see *Nature* 370, 5; 1994), which, among other things, would have opened the way for US participation in the Large Hadron Collider at the European Laboratory for Particle Physics (CERN). The bill passed in the House of Representatives, but not in the Senate, where Senator Bennett Johnston (Democrat, Louisiana) said it should not proceed until the Congress clarifies its approach to fusion research.

The two houses have also been unable to agree on new authorizing legislation for the National Science Foundation (NSF). Like other agencies, the NSF will now have to function without such legislation, taking its direction instead from the appropriations subcommittees that provide its budget.

This week, congressional staff were still working on a reauthorization bill for the National Aeronautics and Space Administration (NASA) which, they say, has a reasonable chance of being passed into law before the session ends.

But other science legislation, including bills authorizing the activities of Environmental Protection Agency (EPA) research, for the possible consolidation of Department of Energy laboratories, for improved risk-assessment processes at the EPA, and for the creation of a National Biological Survey (see below), will not pass through this year's congressional gridlock.

The Clinton administration fought hard last week to salvage a version of the National Competitiveness Act that would pass both houses, but seemed likely to fail. The act contains the research and development programmes to enable the construction of the 'information superhighway'. Legislation on telecommunications regulation, which is considered far more critical to the



superhighway's prospects, was withdrawn last week by a key sponsor, Senator Ernest Hollings (Democrat, South Carolina).

On environmental policy, the setbacks to the administration have been even more severe. Green lobbyists have been stunned by the realization that, even with Clinton and vice-president Al Gore in power, and large Democrat majorities in both houses, they have got nothing through the Congress this year.

The Safe Drinking Water Act, the Clean Water Act, legislation to reinvigorate the Superfund clean-up scheme, and the overdue reauthorization of the Endangered Species Act all seem about to fail. Despite a fierce last-minute push by its industrial and environmental supporters, the Senate is also unlikely to ratify before the adjournment the Biodiversity Treaty agreed at the Earth Summit in Rio de Janeiro in 1992.

Some of the science bills are politically neutral and may be revived next year. But the environmental bills are highly contentious, and their failure this year has left Washington's large environmental lobby in grim mood, particularly as it expects the incoming Congress to be less keen on environmental regulation and more supportive of landowners' rights.

The legislative inaction is leading those who work to influence the law-making process to reopen the question what is Congress for? The voters are expected to answer in their own way on 8 November by rejecting many of the 435 congressmen and 33 senators who are standing for re-election.

Colin Macilwain

Biological survey still left in limbo

Washington. Despite Congress' failure (see above) to pass legislation officially creating a new National Biological Survey (NBS), the survey plans to continue operating within the Department of Interior under the authority of Bruce Babbitt, the interior secretary.

Meanwhile, Ronald Pulliam, the director of NBS, has been telling audiences that the name "survey" has become something of a political liability, and has been stressing the agency's broader role in providing scientific advice on environmental matters to decision-makers.

Following unexpected attacks from supporters of anti-environmental lobbyists in the House of Representatives last year (see *Nature* 367, 400; 1994), the Clinton administration held back from pushing for what NBS officials call "organic" authorizing legislation.

The bill will now have to be reintroduced in the next Congress; without the legal foundation that the bill is intended to provide, NBS supporters fear the agency could be killed by an unfriendly future administration.

Meanwhile, Congress last week approved with little debate \$176 million for

next year's NBS budget in the Department of Interior. The only opposition came in the form of two amendments. One requires NBS researchers to get written permission from landowners before conducting any new surveys on their property, the second prevents the agency from hiring volunteers.

Although the latter restriction could pose a serious obstacle, agency officials may be able to work around it by interpreting the restriction to mean only new volunteers. Volunteers working on existing projects for the Fish and Wildlife Service, from which most of the survey staff were drawn, would therefore still be allowed.

The 1995 budget appropriation, which matches this year's level, allows the agency to get on with the task of setting up pilot research projects as well as strengthening ties with other federal agencies. Last week the NBS signed an agreement to share ecological and biological data with the Environmental Protection Agency and to coordinate the two agencies' research activities, including joint field data collection and integrated ecological risk assessments.

Tony Reichhardt

German parties compete for scientists' vote

Bonn. Germany's ruling conservative coalition of Christian Democrats (CDU) and Free Democrats (FDP) is still odds-one favourite to retain power after the general elections on 16 October. But, whatever the outcome, the chances of a significant change in German scientific research policy are low.

Both the CDU and its main rival, the Social Democrats (SPD) — who could join a coalition with the CDU, with the FDP and the Greens, or even (though less likely), with the Greens alone — are committed to maintaining the strength of German science. The main difference is that the SPD is offering more money (in particular for applied research), while the CDU is holding out for less regulation of researchers.

Both parties want to improve the application of scientific results to new technological developments, and have plans for widening the dialogue between researchers and industry. Both strongly support further development of key technologies such as information and biotechnologies.

Both also agree that research spending by the government should be strictly controlled — which probably means no shift in Germany's uncompromising position that all annual subscriptions to large international research programmes, such as the European Laboratory for Particle Physics (CERN), should be kept constant in real terms. But both are strongly in favour of international research collaboration as a means of sharing the costs of expensive programmes and facilities.

There are some small but significant differences in emphasis. Although it no longer talks of massive funding for solar energy research, for example, the SPD clearly favours socially orientated research programmes, such as health research. In contrast, the CDU is more enthusiastic about industrially orientated research programmes, such as aerospace technologies.

In addition, the parties diverge significantly on two major programmes, both in the planning stage. Because of concern about nuclear proliferation, the SPD would not allow the controversial FRM-II research reactor at the Technical University of Munich to burn highly enriched uranium, as it has been designed to do.

Nor would the SPD back Germany's bid to host the next-generation fusion reactor, the International Thermonuclear Experimental Reactor (ITER), whose estimated costs it regards as excessive.

Overall the SPD has promised that, if elected, it would increase the federal research budget by DM1 billion, bringing it back to the level of the mid-1980s. The party has not said where this money will come from. But Josef Vosen, science spokesman for the parliamentary SPD fraction, says it would be spent on applied research, on the

grounds that basic research in Germany is already well catered for.

In contrast, the CDU has no plans to increase research funding, preferring to concentrate on improving the efficiency with which research funds are used. There is indeed some difference between the two major parties in their views on the appropriate level of regulation of research.

Many German researchers complain about the amount of paperwork they are required to complete, for example when applying for permission to do genetic experiments or to work with animals or radioactivity. Responding to such complaints, the CDU is promising to reduce controls on researchers in order to create a more supportive research environment.



Krüger: unlikely to remain minister.

The SPD claims that it wants to do the same. But, given the tensions between the pragmatic parliamentary fraction of the party in Bonn and the more ideological *Länder*-level groups, its actual position is less clear-cut. Wolf-Michael Catenhusen, head of the parliamentary committee on research, says the need is to maintain regulation while reducing form-filling, and Vosen talks of the need to "de-bureaucratize" laws governing research in genetic engineering.

But the Institute of German Economy, a free-market-orientated think-tank, considers that in practice the SPD will allow ethics committees to increase their influence over researchers, making it harder to reduce the amount of red tape.

In contrast, the SPD may be better placed than the CDU to introduce tax incentives for industrial research. Both parties are in favour of such incentives. But the finance minister in the current CDU/FDP coalition government has consistently resisted the

idea. An SPD-influenced government may push it through faster.

Even if the CDU remains in power, it is unlikely that Paul Krüger, from east Germany, will continue as research minister, as he would probably fall victim to demands on the new government for geographical representation.

Chancellor Helmut Kohl has said that he wants a "leaner government" after the election, with a merging of smaller ministries. This would mean fewer ministerial positions at a time when three of the five new *Länder* in the former East Germany are likely to vote heavily for Kohl, and will expect to be rewarded.

At present, the cabinet has only two east German ministers, both from the state of Mecklenburg-Vorpommern, Angela Merkel, the only woman in the cabinet. Thus Krüger (though not necessarily the research ministry) will almost certainly have to give way to a candidate from either Saxony or Thüringen.

If the FDP fails, as many predict, to reach the 5 per cent of votes required to secure seats in parliament, the chances of a 'red-green' coalition will be higher. Whether researchers should be worried will depend on how the ministries are divided up.

In such a situation, the research ministry would almost certainly go to Peter Glotz, a veteran SPD politician but an unknown quantity as far as science and research are concerned. Better known is the Hessen environment minister, Joschka Fischer, a leading member of the Greens and a strong candidate for federal environment minister.

The flamboyant Fischer has sparked controversy locally: for example, he successfully blocked the building of a production plant for mixed-oxide fuel elements for nuclear reactors (see *Nature* 369, 6; 1994), and introduced speed limits in high-ozone conditions (see *Nature* 370, 321; 1994). He would probably find his influence considerably diluted at federal level, and 'red-green' is probably not as threatening for science as it is often made out to be. **Allison Abbott**

Israel set to join EC Framework programme

London. Israel's scientific community took a step closer to their goal of becoming full participants in European-level research programmes with the decision last week of research ministers of the 12 European Union member states to agree on a negotiating mandate towards this goal.

The mandate established the terms of a broad scientific agreement between Israel and the European Commission in Brussels. Under the terms of such a mandate, the Israeli government would agree

to contribute to the ECU12.0 billion (US\$13.6 billion) Fourth Framework Research Programme.

In return, Israeli scientists would be able to become fully associated with all non-nuclear research programmes under the Framework programme. The agreement will enable Israeli research teams to participate in a wide range of research areas, including biotechnology, renewable energies, biomedicine and advanced materials. □

Discord over IPCC meeting reopens climate dispute

London. Efforts to forge a scientific consensus on the need for measures to combat global warming are threatening to fall into disarray in a dispute over efforts to produce a statement on future climate change that is both scientifically sound and politically acceptable.

At the centre of the dispute is the meeting held at Maastricht in the Netherlands last month of one of three working groups of the Intergovernmental Panel on Climate Change (IPCC), the scientific body set up in 1988 by the World Meteorological Office and the United Nations Environment Programme (UNEP) to collate data on global warming and its likely implications.

The Maastricht meeting produced a 'policymakers' summary' of a special report by Working Group I on the impact of the carbon cycle on climate change. The summary provided new data on radiative forcing — the effect of greenhouse gases on the Earth-atmosphere energy balance — that reinforce the IPCC's earlier conclusions. These formed the basis of the Climate Change Convention signed at the Earth Summit in Rio de Janeiro in 1992, on the need to reduce carbon emissions to avoid a rapid build-up of carbon dioxide in the atmosphere (see *Nature* 371, 274; 1994).

But the US State Department, prompted by energy-industry lobbyists, is said to be planning to send a letter to both Bert Bolin, the Swedish climatologist who is chairman of the IPCC, and Sir John Houghton, former director of Britain's Meteorological Office and chairman of the working group, expressing concern over procedures followed at the Maastricht meeting.

In particular, US officials are thought to be worried that, in apparent contravention of the IPCC's agreed procedures requiring the circulation of an underlying report at least three weeks before a meeting intended to accept it, the summary was discussed and agreed by those present in the absence of final drafts of the five individual chapters of the report (see *Nature* 371, 269; 1994).

The main complaint has come from the Global Climate Coalition (GCC), an industry lobby group based in Washington, which has been set up to express concern about the impact on the competitiveness of US industry of limiting carbon emissions.

The GCC's complaints are backed by a group of five scientists, including noted critics of the 'global warming' hypothesis such as Fred Seitz, former president of the National Academy of Sciences, S. Fred Singer of the University of Virginia, Henry Linden of the Illinois Institute of Technology, and Chauncey Starr, founding president of the Electric Power Institute.

Government officials involved in the IPCC

defend the main substance of the working group's conclusions against the critics' charges. They point out that detailed criticisms of the impact of greenhouse gases on global warming were debated at length — and answered — both within the working group, and in the preparation of various government responses to early drafts of its conclusions.

But there is concern among IPCC officials at the possible implications of their acknowledged failure to follow approved procedures at Maastricht. They defend the procedures adopted on the grounds that the work involved in preparing the final versions of the report's chapters took longer than anticipated, and that those responsible for these final versions were present in Maastricht to answer questions about their anticipated contents.

But in its letter to Bolin and Houghton, the State Department is believed to emphasize the importance of respecting the procedures established by governments for the IPCC if its conclusions are to maintain credibility in policy-making circles.

At the Maastricht meeting, representatives of governments, industry and environmentalist groups were given an opportunity to comment on a draft of a summary of the group's scientific conclusions. IPCC officials claim this process is necessary to ensure that the report's conclusions are widely accepted. "This is not just a scientific report, but one that has to make a political impact," says one government official. "One has to get groups and governments to 'buy into' what is decided, and the only way to do this is to let them have a seat at the table."

But the process has itself caused problems with some of the scientists responsible for preparing the working group's report. "It really upset the lead authors, who felt that policy makers were making changes to the conclusions that were not based on the supporting material," says one participant.

Those who support the IPCC's main argument — that global carbon emissions must be reduced if their concentration in the atmosphere is to be stabilized at levels that prevent dangerous climate change — claim that, having failed to win the scientific debate, critics are now using the procedural error to discredit the whole IPCC process.

But they are worried that the errors could allow opponents of carbon emission controls (for example, the governments of oil-producing states such as Kuwait and Saudi Arabia) to reopen the scientific discussion when the full special report is presented to the full IPCC in Nairobi next month. (Neither Bolin nor Houghton could be reached for comment before *Nature* went to press.)

David Dickson

Anti-plague efforts hindered by lack of recent experience

New Delhi. The epidemic of pneumonic plague spreading through India has caught the country's scientific agencies unprepared. The government had stopped producing anti-plague vaccine five years ago, and reagents and anti-sera for diagnosis of plague victims were not in stock.

India also lacked doctors who had handled plague cases and microbiologists who had seen the plague bacillus, while Khorshed Pauri, a leading virologist with the Indian Council of Medical Research (ICMR) says the present epidemics were due to failure of the surveillance system.

IMAGE
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Masks protect against bacillus aerosols.

"After the last human case in 1969, we thought plague would not come back," says G.V. Satyavati, head of the ICMR. "We do not have a single plague expert in ICMR." A major consequence was that many patients were wrongly diagnosed as having the plague, and sent off to infectious diseases hospitals as a result, only to be told four days later that their plague tests were negative.

An offer from the United States to help combat the epidemic was turned down by New Delhi, which says it is confident of handling the situation on its own. Government factories are working overtime producing drugs and insecticides. Supplies of anti-plague vaccine have resumed and case diagnosis has been speeded up with the arrival of test kits from the Centers for Disease Control and Prevention in Atlanta.

Pneumonic plague has so far killed 47 in the west Indian town of Surat, where the epidemic broke out on 18 September, and four people in Delhi, India's capital. More than 1,200 people in six states are suspected to have been infected with the bacillus *Yersinia pestis* directly from people who fled Surat in panic.

In pneumonic plague, infection with the bacteria *Y. pestis* occurs directly through the lungs when cough-generated aerosols are inhaled from highly contagious infected individuals. This facilitates the rapid dissemination of the bacillus. Antibiotic treatment is effective but must be initiated rapidly, otherwise the disease is often fatal.

K. S. Jayaraman

California law will prohibit genetic discrimination

San Francisco. The governor of California, Pete Wilson, has signed into law a bill prohibiting health insurance companies from discriminating against policy holders or applicants on the basis of asymptomatic genetic characteristics. It also defines the onset of a genetic disease as starting with the appearance of symptoms, not in the demonstration of a genetic susceptibility.

Under the law, which was approved on 23 September, insurers will not have access to the results of genetic tests, and will not be able to use information on genetic status to deny coverage or raise rates to somebody who has no disease symptoms. Violation will result in financial penalties.

The new state law, which also prohibits doctors from releasing genetic test results to insurers or employers, is believed to be the strongest such law in the United States because of the breadth of its definition of genetic information and the number of entities it covered.

Individual and group health insurance, small life insurance policies and group life and disability insurance would be included. Insurers could ask for genetic tests only in the case of large life and disability income policies that already require other types of testing.

Paul Billings, associate clinical professor of medicine at Stanford University and an advocate of genetic privacy protection, claims that privacy and insurance protection were necessary to create an environment in

which the full benefits of genetic testing could be realized.

Billings claims that the state took an important step by defining the onset of genetic disease as the start of its symptoms. This could be particularly important as genes are discovered for cancer and heart disease and in late-onset diseases such as Huntington's chorea.

"We are getting so technologically adept at analysing DNA that we don't quite know when illness begins," says Billings. "It's important because people won't have to grow up with the stigma [or] with the great burden of illness their whole lives."

State senator Patrick Johnston, who represents the Sacramento/San Joaquin region and worked for three years as sponsor of the California bill, said he hoped the new law would encourage people to take genetic tests without fear of losing their insurance.

But some are worried that the new law does not go far enough. Joanna Fanos, for example, a research psychologist at the California Pacific Medical Center in San Francisco, says that other forms of protection may be necessary to ensure that the law is implemented appropriately, and that individuals are not pressurized into taking tests. Fanos is a member of a social issues subcommittee of the American Society of Human Genetics that is developing guidelines on testing in children and adolescents.

Sally Lehrman

US urged to apply cancer research

Washington. A lack of support for "translational research" to carry the results of basic research into the realm of patient care is hampering US efforts in the war against cancer, a panel of leading cancer experts has told Congress.

In a report published in Washington last week, a subpanel of the National Cancer Advisory Board, chaired by Paul Calabresi of Brown University, Rhode Island, calls for an extra \$60 million a year to be spent on research aimed at improving the links between the latest laboratory findings and patient care.

The money should be spent through 60 existing clinical cancer centres approved by the National Cancer Institute (NCI) across the country, the report says. It claims that translational researchers — for example, those who move basic research into technology development, or conduct early clinical trials of laboratory findings — are "an endangered species" under the existing funding structure, and that young scientists consider entry to the

field to be "professional suicide".

The report, which was requested by the Senate and House appropriations subcommittees responsible for funding cancer research, also asks for an extra \$180 million for basic cancer research. Congressional staff reacted coolly to the appeal for extra funds, however, noting that their request for the report two years ago had asked only for an evaluation of the use of existing resources.

The report does call for such a detailed evaluation of the existing \$2-billion cancer research programme, and Harold Varmus, director of the National Institutes of Health, has already approved it.

The panel finds that the considerable progress made by cancer researchers has not always been converted into better care, especially among the poor and the elderly. But it says that talk of health-care reform earlier this year was "devastating the war on cancer by denying resources for research and quality cancer care".

Colin Macilwain

Gene advisory group switches focus to ethical issues

Washington. The Recombinant DNA Advisory Committee (RAC) of the US National Institutes of Health (NIH) is to withdraw from vetting protocols for routine experiments with human gene therapy in order to concentrate on broader ethical issues.

Partly in response to complaints from industry that submitting new protocols for experiments similar to those already approved creates too much extra paperwork, and partly at the urging of NIH director Harold Varmus the RAC is to hand over responsibility for approving standard protocols to the Food and Drug Administration (FDA).

Leroy Walters, professor of philosophy at Georgetown University in Washington, and chairman of the RAC, told the House of Representatives Committee on Science, Space and Technology last week that in future, only protocols raising novel scientific or ethical questions would be submitted to his committee.

The RAC voted at a meeting last month both to approve this change in its role, and to become an advisory committee to the FDA as well as to the NIH. Varmus had asked the RAC to consider whether it should continue to review all protocols, or spend more of its time looking at broader ethical issues.

Philip Noguchi, director of cellular and group therapies at the FDA, says that industry will initially see little difference between old and new procedures, but that the reduction in RAC's responsibilities is an important step towards meeting industry's concerns.

According to Noguchi, the change will free the RAC to spend more time dealing with the ethical issues that will arise from human gene therapy.

The RAC was created in 1974 to draw up guidelines for the emerging field of recombinant DNA technology. As it developed the guidelines it tended to become involved with genetic engineering as well. "There were always new experiments testing categories," says to Sheldon Krinsky, professor of urban and environmental development at Tufts University, and a member of RAC in the late 1970s. "It was an inductive way of establishing guidelines."

As the field of genetic engineering has developed, the RAC has gone through various changes, recently the Environmental Protection Agency took over the regulation of large-scale field tests of genetically engineered products. "The RAC has been straying from its original purpose, and these changes take it back to its original intent," says Alan Smith, chief scientist at the biotechnology company Genzyme.

The proposed changes are supported by both Varmus and David Kessler, head of the FDA. They are expected to come into effect at the end of the year. **Helen Gavaghan**

Europe opens new era in synchrotron research

Grenoble, France. Future prospects for research into the structure of matter took a major step forward last week with the opening in Grenoble of the 6-GeV European Synchrotron Radiation Facility (ESRF) which extends the wavelength range of synchrotron radiation from 'soft' to 'very hard' X-rays.

The FFr3.6-billion (US\$681-million) ESRF is the first of the so-called third-generation synchrotrons, which produce fine and brilliant beams of X-rays at chosen wavelengths — almost an "X-ray laser", says Yves Petroff, director general of ESRF. The United States will also open a 7-GeV machine (the Advanced Photon Source) at Argonne in 1996-97, and Japan an 8-GeV machine, the SP-8, at Kobe a year later.

Synchrotron radiation was first observed in 1947 by researchers at General Electric in the United States. They found that each time the trajectory of high-energy electrons in a circular accelerator was bent by magnets, the electrons emitted a cone of electromagnetic radiation, similar to the cone of light from the headlights of a car turning a bend.

Initially, scientists saw this synchrotron radiation as simply a curious nuisance that reduced the energy of electrons in particle

accelerators. Only in the 1960s did scientists recognize the potential of synchrotron radiation for overcoming the problems of the conventional Coolidge X-ray tube, which emits relatively feeble and direction-less X-rays that are intense only at a few wavelengths, and cannot be focused or polarized.

First-generation synchrotrons amounted simply to crude X-ray beams clipped on to existing particle accelerators. What sets ESRF apart from previous purpose-built synchrotrons — such as the second-generation 2-GeV machine at the Daresbury Laboratory in Warrington, in the United Kingdom — is its threefold higher energy, the consequent ability to attain wavelengths thousands of times shorter, and the much higher (up to five orders of magnitude) brilliance of its X-ray beams.

ESRF's high energies are obtained by first accelerating electrons to 200 MeV in a linear accelerator, and then to 6 GeV in a

IMAGE
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Philippe Piquet / Eurisys/SPL

The ESRF: 'almost an X-ray laser'.

300-metre-circumference synchrotron. The electrons are then fed into the 844-metre-circumference storage ring (see photo).

Here we find the big third-generation innovation — the addition of 'insertion devices'. Existing synchrotrons produce radiation only when high-energy electrons pass bending magnets in the storage ring.

But ESRF also produces radiation at 29 insertion devices, each of which creates an oscillating magnetic field that forces electrons to slalom about the linear part of their trajectory.

The result is a series of X-ray beamlines that shoot off from the storage ring at tangents, arranged like multiple exits on a motorway roundabout.

Here big science ends, and small science begins. Outside research groups simply take a beam, refine it by passing it through a slit, choose the wavelength they need using a monochromator, and focus the beam onto their sample using a mirror.

Indeed, ESRF will do little in-house research. Four-fifths of user time on public beamlines is reserved for free use by outside research groups. Nine such public beamlines are already installed, and two more are planned by the end of the year; 101 groups have been selected from around 300 applications by peer review for the first trimester of operation.

ESRF has a capacity of 50 beamlines. In addition to the public beamlines, 24 beamlines will also be built and paid for by groups of research institutes — Collaborative Research Groups (CRG) — at a cost of between FFr10 million and FFr20 million each. ESRF will be entitled to hire out one-third of user time on CRG beamlines.

More than 6,000 European researchers now use synchrotron radiation, says Ron Newport, chairman of the ESRF council and head of the Daresbury Laboratory. The list of peer-review committees gives some idea of the range of ESRF's applications — hard condensed matter, soft condensed matter, chemistry, life sciences, surfaces and interfaces, and methods and instrumentation.

Declan Butler

ESRF as 'a model of collaboration'

Grenoble, France. Built on schedule, within budget and above specification, the European Synchrotron Radiation Facility (ESRF) is a model of how to do big science, according to François Fillon, France's minister of research and higher education.

Fillon would like to see the "pragmatic" ESRF model extended to other big science projects, with different countries contributing according to their scientific and technological needs.

European science, he said at the opening ceremony of the ESRF in Grenoble last week (see above), needs to avoid locking itself into an "overformalized institutional approach", which often creates as many problems as it solves.

Aware of Europe's failure to present a united face to its potential partners in the Large Hadron Collider at CERN, the European Laboratory for Particle Physics, Fillon also says that Europe should negotiate *en bloc* on international projects. He would like Europe's science ministers to hold annual meetings on big science projects in order to reach a common position.

One reason for ESRF's success, says Ron Newport, chairman of the ESRF council, is that participants "volunteer" and negotiate their contributions according to "perceived needs and not some predetermined formula".

Yves Petroff, director general of ESRF, says that the security of multi-annual budg-

ets is essential. The facilities' members agreed to provide FFr2.2 billion (US\$416 million) from 1988 until mid-1994 to cover initial construction costs, and a further FFr400 million for construction and FFr1.06 billion for running costs from mid-1994 until 1998.

But in contrast to the United States and Japan, where private companies are planning to pay much of the costs of the proposed third-generation synchrotrons, European companies have no plans to build their own beamlines at ESRF. "We hope this will change", says Petroff.

Despite this lack of corporate funding, Newport points out that European industry need not lose out on benefits from ESRF, as it will still be able to buy time on the facility. ESRF will charge private users FFr3,000 per hour if they publish their results, but will make a surcharge for confidentiality. It will also offer a full contract research service.

FFr1 billion of the FFr2.2 billion costs of the first phase of ESRF construction has already gone into the pockets of European companies, says Newport. In addition, the sophisticated engineering developed at ESRF will be licensed; new optics had to be developed, for example, because conventional optics could not cope with the high-power density of the X-ray beams, namely several kilowatts per square millimetre.

Declan Butler

Preservation of rice strains

SIR — K. S. Jayaraman's article, "India set to end 'gene robbery'" (*Nature* 370, 587; 1994) ends by saying: "India has also taken steps recently to get back some of the materials it has already lost. Two months ago, the International Rice Research Institute [IRRI] in Manila in the Philippines agreed to return to India 5,000 accessions of traditional rice strains from Assam state, which were taken away by the institute in the 1960s."

This is simply a repeat of allegations of "gene robbery" by IRRI made in an article in the *Illustrated Weekly of India* on 23 March 1986. One month after the article appeared, the Rice Research Workers of India were reported to have "unanimously condemned such gross distortion of facts in the article at their annual meeting in Faizabad", and in its issue of 29 June 1986 *Illustrated Weekly* published a four-page rebuttal of the accusations by Dr M. S. Swaminathan, then director general of IRRI.

The traditional rice strains were not "taken away" by IRRI in the sense that they were removed arbitrarily or illegally. These rice strains, or germplasm, known as the Assam Rice Collection and containing more than 5,000 traditional rice cultivars, were collected 30 years ago in Assam and sent by the Indian Council of Agricultural Research for safekeeping at the IRRI genebank in Los Baños, Philippines, precisely to preserve the diversity of the rice gene pool.

"The collection included native diversity in rice available at that time in remote areas, providing valuable genes for resistance to some serious insect pests", said Dr R. S. Rana, director of India's National Bureau of Plant Genetic Resources, in a letter to IRRI, requesting that the institute duplicate the collection and send it to India because, he said, much of the original collection is no longer available because of "deterioration or loss".

The collection in IRRI's Genetic Resources Center rice genebank is a duplicate of the original Assam Rice Collection. Beginning in August, about 50 kg of the Assam rice seeds will be sent to India in two batches representing more than 5,000 accessions.

The IRRI rice genebank holds in trust more than 80,000 different samples of rice varieties and wild species of rice from all over the rice-growing world. Since 1973, more than 740,000 packets of rice seeds have been distributed, free of charge, to rice scientists worldwide for use in research.

Rice seeds from many countries have contributed useful traits to modern rice varieties, such as early maturity, resistance to diseases and pests and tolerance for salinity, flooding and submergence,

which have benefited mostly Asian countries.

IRRI has repatriated seeds to several countries, including the Philippines and Sri Lanka. In one case the institute helped to reintroduce to Cambodia rice seeds that had disappeared during the long civil war in that country. The lost varieties, collected in the early 1970s in Cambodia and stored in IRRI's rice genebank, are once again being grown extensively in that country. IRRI last month repatriated to Indonesia and Thailand 416 and 392 samples respectively.

M. Jackson

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Combating AIDS

SIR — Is it really true, as you say, that "the message about the dangers of AIDS is not getting through"?¹ Numerous studies, even among disadvantaged groups^{2,3}, show that knowledge about transmission routes is very high, but there is a hard core of people familiar to clinicians (perhaps 35 per cent of the total population at risk), who may resist counselling⁴ in spite of their best endeavours or fail to modify their risky behaviour⁵. The most successful approach so far seems to be small group therapy^{5,6} bearing a surprising resemblance to Alcoholics Anonymous groups.

Although the present situation may tempt us to despair, this may be historically unwise. After all, 70 years ago, the orthodox medical opinion similarly was that what we now call alcoholism was utterly incurable.

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Not predisposed

SIR — Under the heading "A predisposition to astronomy" (*Nature* 368, 800; 1994), you say: "Astronomy in Mexico was well established when, in 650 AD Maya, Zapotec and Theotihuacán

astronomer-priests met at the hilltop observatory of Xochicalco, Morelas, to synchronize their sundials. This Mesoamerican Calendrical Convention is arguably the first scientific conference on record — the poster session is still carved into the base of the pyramid."

An examination of the base and other parts of the pyramid shows no description of a convention nor of a poster session. Nothing indicates that a calendar was synchronized in Xochicalco (Mexico) nor in Copan (Honduras), nor elsewhere in Mesoamerica.

Jean-Pierre Herveg

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Alas, poor Yorick

SIR — The account by Michael Fitzgerald and David Berman (*Nature* 368, 92; 1994) of the illness of my cousin Yorick Smythies is completely erroneous. He did not suffer from paranoid schizophrenia as they claim he did. From information I received from Yorick himself and from his wife it is clear that his original symptom was depression for which his doctor unfortunately prescribed amphetamines to which Yorick became addicted (as commonly happened) and subsequently he developed a wholly iatrogenic chronic paranoid amphetamine psychosis.

J. R. Smythies

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TV's disservice to science

SIR — I wholeheartedly agree with your criticism of the BBC's appalling 'Heretic' series, but I feel that the independent television companies do an even greater disservice to science, engineering and the enquiring mind among the young in Britain when they broadcast a series of advertisements suggesting that it is "sad" (a word the young construe somewhat differently from those over 30) to want to know how the "widget" in a can of beer works.

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Brazil's best university seeks fame

The University of São Paulo believes it should be making a more prominent mark on the world outside Brazil, but the difficulties of doing so from within a still-developing country are greater and less tangible than may be thought.

São Paulo. This city, the traffic-jam capital of the world, may not be the whole of Brazil, but it is more than 10 per cent of it in terms of population, while the state of which the city is the capital earns roughly half of Brazil's gross national product and is home to half of Brazil's research.

An industrial city, São Paulo has managed to keep many patches of elegance and greenery among the high-rise buildings and football stadia; the cardboard slums are inconspicuously on the periphery. The city also houses the largest of the University of São Paulo's five campuses, with some 30,000 undergraduates on the site.

Like universities throughout Brazil, the University of São Paulo (USP) is a young institution — and looks and feels like that. It was formed only in 1934, around pre-existing and free-standing schools of law, engineering and medicine. Unlike others among Brazil's universities that are known elsewhere, São Paulo is proudly and advantageously a child of the state government, whose constitution requires that a fixed proportion (about 9 per cent) of its revenues should be spent on higher education.

Once that cake is cut each year — the other chief beneficiary is the University of Campinas — the university is financially autonomous, but the new vice-rector wistfully observes that 85 per cent of the state's contribution to the annual budget goes on salaries, and much of the remainder on heat, light and the like. Research is financed separately, partly by contracts with government agencies or industrial companies (multinational rather than Brazilian) and partly by means of competitive research grants from the state-run research foundation whose budget is again a fixed proportion (roughly 1 per cent) of the state's spending.

Never have academics sung so warmly the praises of a state-run research foundation as this university's do for the state of São Paulo's research foundation, FASESP. Five years appear not to have abated admiration for the foundation's way of working (see *Nature* 342, 372; 1989). The foundation works quickly, using academics rather than paid officials to make decisions, decisions are respected even by those who are refused funds and hardly any of the \$100 million a year is spent on administration. By comparison, getting funds out of the national research foundations is like pulling paper tissues out of a water-soaked box. And the São Paulo campus of USP reckons routinely to capture half the total.

So why does this university, by general agreement the best in Brazil, have a palpable sense of disappointment at its standing among the world's universities? "Tell us why", demanded one of half a dozen physicists last week, "you think that São Paulo is the best?" References to São Paulo's predominance among the trickle of Brazilian submissions to this journal, and to the frequency with which Brazilian visitors elsewhere turn out to be from this university, fail to convince. The university would give its eye-teeth for a reputation for excellence in some field.

It is a curious dilemma. Brazil is hardly a typical developing country. As in India, of course, there is a cruel contrast between the rich (some of whom are seriously rich) and the poor. But, by contrast with India, academics seem united in holding that the poor must quickly be made prosperous. (One of them, Professor Isaias Raw, director of the Butantan biotechnology centre, distinct from but adjacent to the São Paulo campus, says that Brazil owes this duty even to the primitive Indian communities of Amazonia.)

So whence the vague sense of isolation? Not isolation from the Internet, certainly. Those who are so inclined can hook up as easily as anywhere, and proudly proclaim their e-mail addresses on the name-cards. (The suffix ".usp.br" will get you to São Paulo.) Nor can it be that active researchers fail to attend meetings overseas. São Paulo's research foundation is generous with travel grants. And most people's English is excellent, while the Latin roots of Portuguese are easily recognizable.

Part of the explanation is historical, and recently so. The long period of military rule (between 1962 and 1982, with a kind of transitional period until 1985) has scarred the universities. It is not simply that many able people were forced out of their jobs (Isaias Raw first read of his dismissal in the newspaper), but that those left behind in the departments were induced by fear not to make difficulties of any kind, among which over-intimacy with the world elsewhere could easily have been one.

This was also a period in which the military government embarked on nuclear and space research programmes quite separately from the academic community. This is when, according to one last week, the academic community in Brazil as a whole "was isolated from the people". The political turmoil of the Collor presidency, brought prematurely to an end two years ago by charges of embezzlement, has not helped.

The university's own constitution is a further difficulty. All academics are tenured. Autonomous though the university may be, while those who have tenure and no great zeal for research are in the majority, São Paulo will be struggling to build depth around its research programmes. There are further problems about appointments; departments recommend the appointment of new recruits, but the university has the last word and does not always grant departments' wishes.

Even so, tough questions are being asked. An evaluation of all 70-odd departments and institutes has been mounted. There is also a project to define what is meant by a "department" that promises more radical criteria for the future division of the university's income; São Paulo seems on the way to rediscovering the general rule that the smaller a department, the more expensive and less productive it tends to be.

The more serious difficulties are to do with questions of critical mass. Active parts of the university are excellently equipped, while academics are well (if too uniformly) paid. (Salaries range from US\$1,500 to \$5,000 a month, including a federal supplement of the salaries of senior people.) The physics department boasts of no fewer than 17 buildings, enclosing a splendid low-temperature facility and a small tokamak, for example. But there is rarely a great enough concentration of people in one field of research to keep up with the furious pace of discovery elsewhere in the world.

Walter Colli, director of the Institute of Chemistry, sees the problem clearly. The institute has enough people to make a mark. By concentrating on fields such as protein structure/function relations, the physiological role of free radicals and chiral organic synthesis, he plans that those concerned should make their mark within the coming 15 years. It is a brave hope, but one echoed by many others at São Paulo last week.

Isaias Raw is in a greater hurry. The Butantan Institute was meant, at its foundation in 1901, to be Brazil's equivalent of the Pasteur Institute. Since 1985, it has been a biotechnology centre as well, but has had to recreate the technology of making snake-venom antisera. If, after the general election this week, Brazil adopts a law conferring patent rights on biotechnology processes developed elsewhere, Raw will have only a 12-month grace period in which to develop Brazil's equivalents. It may be true, as one academic put it last week, that "Brazil is too big to be a small country", but old habits die hard.

John Maddox

Arctic chill for CO₂ uptake

Christopher B. Field

MOST of the world's plant species respond to increased atmospheric carbon dioxide with increased photosynthesis and decreased water loss, at least in the short term and at the single-leaf level. But a leaf on a plant in a pot is an incomplete and potentially misleading representative of an ecosystem, especially in the longer term. In three-year experiments with natural Arctic tundra, described on page 500 of this issue¹, Oechel and colleagues doubled the CO₂ concentration in greenhouses, combining this in some cases with a temperature increase of 4 °C. The message is that, in this ecosystem, extra CO₂ alone does not boost carbon storage in the long term, although a warmer climate plus extra CO₂ may do so.

The challenge of understanding the responses of ecosystems to increased atmospheric CO₂ has several interacting dimensions. Agriculturalists are chiefly interested in crop yield and nutritional value; ecologists, in the effects on primary production and the global cycles of carbon, water and nutrients, and in knock-on effects on the distribution of grasslands, forests and deserts. Oechel and colleagues address some of the ecological issues but concentrate on the future composition of the atmosphere. Their focus is on net ecosystem carbon balance — storage or loss of carbon from all plant and soil pools. In their greenhouse-enclosed systems in north Alaska, they found that although both experiments receiving increased CO₂ initially stored additional carbon, the response in the plots without the temperature increase gradually decreased and was gone by the third growing season. The increased carbon storage in the combined CO₂-temperature treatment persisted for the three years of the experiment.

The net ecosystem carbon balance summarizes the local impact of ecosystems on atmospheric CO₂. A large storage or sink indicates that ecosystems will mop up some of the carbon released from fossil fuel combustion and other anthropogenic sources. The absence of a persistent sink implies that ecosystem processes can do little to put the brakes on the atmospheric increase, leaving the burden to the oceans and to society.

Currently, the annual increase of atmospheric carbon (about 3.4 Pg, or 3.4×10^{15} g, of which more than 99 per cent is in CO₂) represents about 48 per cent of carbon inputs from the sum of industrial emissions (primarily from fossil fuel combustion and cement production) and tropical deforestation². The fate of the carbon emissions that do not remain in the atmosphere is central to the understanding of the global carbon cycle, to our

ability to predict future levels of atmospheric CO₂ and to the credibility of policies for limiting its increase.

The world's oceans are one main sink, generally thought to take up about 60 per cent of the total (although it could be substantially less³). Additional carbon is sequestered in terrestrial ecosystems, driven by an incompletely quantified combination of increased CO₂, fertilization by

IMAGE
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REASONS

Bleak outlook — Arctic tundra is unlikely to mop up excess carbon dioxide.

nitrogen and other mineral elements, regrowth of forests in previously deforested regions, and climate factors. An idea of these terrestrial sinks can be gained from model calculations⁴ or direct measurements under current conditions⁵, but it is difficult to extrapolate these to a higher-CO₂ future. Experiments that impose an artificial future climate or atmosphere are much more definitive.

But such direct estimates are also much more local and may not be representative of patterns across the broad range of climate conditions and ecosystem types. From this, the first CO₂ fertilization experiment on the ecosystem scale, the foundation for generalization is necessarily incomplete. Yet specific aspects of the Alaska results, combined with related studies, are beginning to yield general patterns. Central to these is the accumulating evidence that, at the scale of the complete ecosystem, indirect responses to increased CO₂ can be at least as important as the direct responses of photosynthesis and transpiration. The specific indirect responses appear to vary from ecosystem to ecosystem, but the variations look increasingly systematic.

On the tundra, Oechel and colleagues found that the dominant indirect effect of increased CO₂ was to decrease photosynthetic capacity. In the short term, rates of CO₂ fixation were stimulated by increased CO₂ (ref. 6), but this stimulation disappeared within a few weeks⁷. With photosynthesis per unit of leaf area similar in plants at raised and at ambient CO₂, the primary driver for the initially increased carbon storage in the elevated CO₂ experiments was a lengthening of the season for strong sink activity rather than increased photosynthesis⁸.

Compared with many other terrestrial ecosystems, plant growth in Arctic tundra is strongly limited by low temperature and low nutrient availability, both of which could limit the response to extra CO₂. The role of low temperature is indeed implicated in the results from experiments combining a CO₂ increase with a warming of 4 °C. But the gradual decrease in net carbon storage in the experiment with extra CO₂ alone suggests another indirect effect. As plants cannot build tissue without simultaneously using nutrients, increased carbon storage in one year should lead to decreased nutrient availability the next year, decreasing the potential for continued carbon storage, especially in ecosystems where elevated CO₂ does not lead to large changes in the nutrients required to produce a unit of plant tissue.

A nutrient connection may also explain the positive response of carbon storage to the combination of increased CO₂ and increased temperature in these experiments. The warmth may stimulate nutrient availability through increased decomposition of nutrient-rich soil organic matter. Does this mean that substantial carbon storage is more likely in a global change scenario that includes both warming and increased CO₂? Maybe, but elevated temperature increases nutrient availability largely by stimulating decomposition — carbon loss from the soil. An altered balance between increased carbon loss from the soil and increased plant growth resulting from extra nutrients should equilibrate at some point, and even a three-year experiment provides little insight into when.

Over the broad range of terrestrial plants and ecosystems, complete down-regulation of photosynthesis to the point where CO₂ uptake rates are the same in plants grown at ambient and doubled CO₂ seems to be the exception rather than the rule. Other indirect mechanisms that could limit carbon storage include genetic constraints on plant responses and nutrient limitation, which could be exacerbated by the slower decomposition of plant tissue grown in a CO₂-rich atmosphere. On the other hand, indirect effects of increased CO₂ on soil moisture or nitrogen fixation could stimulate carbon storage. Where water is scarce, indirect

B & C Alexander

effects of CO₂ on soil moisture can lead to increased plant growth, especially in dry years⁸. But this does not necessarily imply increased carbon storage, which is controlled by the balance between plant growth and decomposition — under many circumstances, decomposition tends to increase in parallel with plant growth.

Oechel and colleagues found no evidence for long-term stimulation of net ecosystem carbon balance by increased CO₂ alone. Yet theirs was not really a long-term study from the perspective of the lifespan of the tundra plants or the dynamics of global change. Even the complete homeostatic adjustment of net ecosystem carbon balance at higher CO₂ might be an artefact of the study's limited duration or of the climate during those particular years. Clearly, though, increased carbon storage is not an automatic consequence of ecosystem-level exposure to increased CO₂. Arctic tundra is unique in many respects. Increasingly, it is possible to predict ecosystem responses to

global change, accounting for unique features of different ecosystem types. Long-term, ecosystem-scale experiments provide unparalleled opportunities for testing these predictions and for improving the understanding necessary to make better predictions in the future. □

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VISION

When it pays not to see

Michael J. Morgan

DURING rapid eye movements, for example those made while scanning this article, we are not normally conscious of the smear caused by movement of the image over the retina. On page 511 of this issue¹, a group of workers at Pisa and Perth clear up some confusion by showing that this 'central anaesthesia'² of vision during eye movement is selective for motion signals.

Eye movements are important in compensating for the poor spatial resolution of our vision outside of the central area (the fovea), but they bring with them a potential inconvenience. During each saccade the retinal image is displaced at several hundred degrees per second, and it is easy to show by psychophysical experiments that such displacements of the image are perceived as movement if they occur when the eyes are stationary. Therefore our vision should be continuously interrupted during eye movements by annoying motion signals, which would interfere with our perception of a stable, external world.

The suggestion that vision is suppressed during saccades was made by Dodge as early as 1900³. The experimentally inclined reader can observe this suppression by looking in a mirror and changing fixation from the image of the pupil to the edge of the eye. The eye movement is invisible in the mirror, but this is not because the movements are too small or too fast to be seen: they are easily observed when looking at another person's eyes.

It appears that perception is interrupted

during a saccade. But measurements of the extent of suppression have given contradictory results, and there are many cases where vision is manifestly clear during saccades. For example, when looking at the track from a fast moving train, the sleepers become visible only when we make a saccade against the direction of the train, and thus briefly stabilize the image of the track on our retina. A light-emitting diode viewed in a dark room will appear as a series of dots during a saccade, each dot corresponding to one flash of the diode. Why has suppression failed in these cases?

Burr, Morrone and Ross¹ cleverly show that the suppression is confined to one class of perceptual mechanism, involved mainly in the signalling of motion. The neural substrate of this mechanism is probably the magnocellular pathway leading from a class of ganglion cells in the retina having relatively large receptive fields, and high contrast sensitivity (variously and confusingly called the 'parasol', 'M-cell' and 'P-alpha' group). This pathway is anatomically distinct from the parvocellular pathway, which has smaller cells ('P-beta') of lower contrast sensitivity, at least up to the main input layer (IVc) in the primary cortical visual area V1. A key difference between the M- and P-pathways is that only in the latter are neurons selective for the wavelength of light. The P-pathway thus provides the substrate for our perception of colour. In the M-pathway, on the other hand, the signals from the different cone classes

appear to be randomly combined, so there is no systematic colour signal.

It is possible to devise stimuli that are (at least in theory) visible to the P-pathway because they contain differences in hue, but not to the M-pathway because they have no differences in white-black contrast (luminance). Burr *et al.* show that such 'equiluminant' stimuli are essentially unaffected by saccades. They are as visible when flashed during a saccade as when flashed before or after. The P-pathway is also thought to be responsible for detecting high-spatial-frequency stimuli; in other words, those that can be seen only when the image is in sharp focus. These too are little affected by saccades. On the other hand, coarse gratings containing no hue are suppressed during saccades. These are exactly the kinds of stimuli that would be detected by the M-pathway.

The idea that the M-pathway is selectively suppressed during saccades is supported by a study in which the variation in contrast detection thresholds with wavelength was measured for stimuli presented briefly either during or before a saccade⁴. Thresholds during a saccade showed the sensitivity to wavelength expected of the P-system; those presented outside the saccade were more characteristic of the M-system.

The only puzzling feature of the Burr *et al.* study is the hint of an enhancement of sensitivity to colour during saccades. In interpreting this finding it may be relevant that the P-pathway probably carries both an achromatic and a chromatic signal⁵. Perhaps a saccade destroys the notional equiluminance of the chromatic grating, either for mechanical or neural reasons, thereby making the target more visible.

Why should the M-pathway be selectively suppressed during saccades? The image during a saccade is moving at high velocity, and probably provides an input mainly into the coarse and relatively rapidly acting M-pathway. The P-system may hardly be active during a saccade and so nature, ever economical, has not bothered to suppress it. Bishop Paley would have seen this as another wonderful example of the cleverness of The Designer, and Darwinists will see it as a triumph for the blind watchmaker. In this case, however, it is not only the watchmaker who is blind, but the M-system during saccades. □

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Drawing a double-edged sword

Harvey R. Colten

WHILE cruising the Mediterranean aboard the Prince of Monaco's yacht, Charles Richet and Paul Portier began a set of experiments that led to their discovery of anaphylaxis, a sudden devastating immunological reaction, and to the Nobel Prize in Physiology or Medicine for Richet in 1913 (see illustration on next page). The prevailing wisdom at the time was that immunization with small amounts of toxins gave protection against any future encounter (phylaxis), so they were surprised to find that animals previously exposed to sublethal quantities of the toxins from the jellyfish *Physalia* or sea anemone *Actinaria* were so sensitive to the toxin that they died shortly after receiving even a tiny dose¹. Within a year Arthus described what he believed to be a cutaneous manifestation of anaphylaxis resulting from repeated intradermal injections of horse serum². The Arthus reaction and anaphylaxis were later shown to be quite distinct pathophysiologically,

however, and to be examples of a much broader set of immunological reactions. These were early indications that inflammation is a dual weapon — one that is critical for host defence, but also a potent trigger of serious, even fatal, disease.

Ninety years on from the observations of Richet, Portier and Arthus, the techniques of modern biology are being employed to dig deeper into the complex systems that mediate inflammation. A spate of papers³⁻⁶ over the past months attests to the level of interest in the problem. All of them employ targeted gene disruption by homologous recombination. And all of them bear on what I see as a central issue in this field. Is inflammation primarily triggered by cellular immunoglobulin Fc receptors or by complement activation?

In these new experiments, deletions of cell-surface receptors for immunoglobulins³⁻⁵ or deletions of a specific class of immunoglobulin⁶ were used to define

the essential components of hypersensitivity reactions. Receptors for immunoglobulin on mast cells (and on other cells of haematopoietic origin) are multimeric complexes derived from several genes. So by targeting the common subunit γ -gene, Sylvestre and Ravetch³ and Takai *et al.*⁴ were able to delete both IgE Fc (Fc ϵ RI) and IgG Fc (Fc γ RI, Fc γ RIII) receptors; likewise, Dombrowicz *et al.*⁵ selectively deleted the IgE Fc receptor by targeting its specific α -chain. In both strains of mice, anaphylactic reactions to systemic or cutaneous antigen stimulation were abrogated or markedly attenuated.

In contrast, Oettgen *et al.*⁶ showed that antigenic challenge of mice deficient in IgE immunoglobulin led to systemic anaphylaxis indistinguishable from that observed in wild-type mice. These data back up earlier work^{7,8} that defined the IgG immunoglobulins as capable of triggering anaphylaxis. Among the IgG antibodies that generate this hypersensitivity reaction are some that activate the complement cascade and others (non-complement fixing) that bind to tissue mast cells⁷. That the latter subclass of antibody is more important in systemic anaphylaxis is suggested by Oettgen and colleagues' finding that complement depletion of the IgE-deficient mice had little or no effect on the response.

Sylvestre and Ravetch³ showed that the Arthus reaction is also markedly attenuated in animals deficient in the Fc γ R chain, and that complement depletion abrogates the residual inflammatory response. Ravetch⁹ has compiled results from these studies to develop a concept of the 'primacy' of Fc-receptor interactions with immune complex (antigen-antibody) which triggers the subsequent cellular/humoral events of inflammation. In his view, complement has a secondary role by amplifying the Fc-triggered response.

This stands in contrast to the idea that immune-complex activation of the complement cascade is the main event triggering IgG-mediated hypersensitivity reactions. As outlined below, the relative importance of these two mechanisms is a function of the immune status of the organism. Activation of complement generates complement-derived polypeptides (such as C3a, C3b, C3bi, C5a) that interact with their cognate cellular receptors. The result is increased vascular permeability, smooth-muscle contraction, altered expression of cell-surface receptors, directed migration and mediator release from leukocytes, all components of inflammatory reactions.

Oettgen *et al.* conclude that complement plays little or no part in these IgG (non-IgE)-mediated hypersensitivity reactions by 'depleting' complement in their knockout mice with cobra venom factor. Sylvestre and Ravetch also compare the effect of cobra venom depletion

Cold comfort in Chicago



THIS life-sized reconstruction of a mammoth-bone hut is just one of the spectacular items on show at *Teeth, Tusks and Tar Pits*, a permanent exhibition opening on 12 November at the Field Museum of Natural History in Chicago. The exhibition adds thematically to the *DNA to Dinosaurs* hall which opened on 11 June, taking the history of life from the end of the Mesozoic 65 million years ago to the present day. As well as the mammoth hut and several other ice-age exhibits appropriate for a city in the northern United States, *Teeth, Tusks and Tar Pits* features the minutely detailed fossils from the Eocene Green River formation of Wyoming. Displays on the evolution of humanity will excite particular interest, given the prominence of creationist remarks in the (admittedly self-selected) sample of comments pinned by visitors to bulletin boards at intervals throughout *DNA to Dinosaurs*. The two exhibitions have a combined area of 23,000 square feet, so visitors are advised to wear comfortable shoes and be prepared to take a whole day to get around them.

H.G.



Commemoration, in philatelic form, of the discovery of the phenomenon of anaphylaxis by Richet and Portier.

of complement and FcR γ deletion to conclude that the IgG Fc receptor is the quantitatively more important trigger of the Arthus reaction. Cobra venom factor is the homologue of a cleavage product of the mammalian complement C3 protein (C3b) that resists inactivation by endogenous control proteins. Hence, its administration *in vivo* results in wasteful complement activation and a marked but transient decrease in serum complement (C3). This experimental trick, and the use of mice genetically deficient in the next protein in the complement cascade (C5), had previously defined an important role of the complement system in anaphylaxis, the Arthus reaction and other aspects of the immune response¹⁰.

At least two factors must be considered in reconciling these experiments that define the relative role of IgG Fc receptors and complement in hypersensitivity reactions. First, complement within the vascular space (though easily measured) is not the most important source of these proteins for the early events leading to inflammation; second, the relative involvement of the complement system and Fc receptors as primary triggers of inflammation depends on immunological experience; that is, complement is essential for a response, especially in the immunologically immature or naive.

The complement proteins in serum are almost entirely of hepatic origin but many extrahepatic sites of complement gene expression have been identified. Indeed, active tissue injury, infection and so on elicit quantitatively greater changes in

complement production at these extravascular tissue sites than in hepatocytes¹¹. These extravascular sources of complement are believed to be critical in the earliest phases of inflammation. Recruitment of cells and soluble constituents from the vascular compartment are later events in the response. The effect of cobra venom factor on this extravascular source of complement is unknown. Mice in which the complement C3 gene has been deleted

will become available before too long, and allow a more critical assessment of this issue.

Complement is clearly implicated in host defence and hypersensitivity responses in the immunologically immature host. For instance, exhaustive studies of passive cutaneous anaphylaxis in C5-deficient and C5-sufficient mice¹⁰ show that, at low concentrations of antibody, no anaphylactic reaction can be elicited in the complement-deficient animals. When antibody is supplied in high concentrations this dependence on complement is relieved. Likewise, the importance of complement in specific immunity (quantitative antibody response and isotype

switch from IgM to IgG antibody) in complement-deficient animals is manifest only at low antigen doses¹². The conditions of limited antigen and antibody mimic the initial encounter of an immunologically virgin or minimally sensitized animal with a foreign antigen. Finally, it would appear that the answer is similar for non-immune tissue injury; for example, the size of experimental myocardial infarcts can be limited by blocking complement effector proteins, which in turn prevents inflammation-mediated propagation of tissue injury¹³.

Is the distinction between Fc receptors and the complement system as primary triggers of inflammation merely of academic interest? I think not, for the development of strategies to prevent or interrupt adverse immunological events, while retaining immune-mediated host defences, is a highly desirable therapeutic goal. It will be brought closer by the refinement of the Fc-receptor- and complement-triggered models of inflammation. □

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OPTICAL MATERIALS

Polymers scale new heights

W. E. Moerner

THE photorefractive effect first manifested itself as a nuisance, a lingering inhomogeneity in the refractive index of crystals that had been used for nonlinear optical experiments. Now, though, it is seen as a promising way of producing holograms for optical processing and information storage. Photorefractive polymer materials are attracting increasing interest for such technological applications, and the remarkable performance of the material now described by Meerholz and colleagues on page 497 of this issue¹ brings the dream several steps closer to reality.

How the effect works is described in detail in the accompanying box. In brief, a pattern of light intensity (say, from two interfering light beams) produces charge redistribution within the material that modulates its refractive index. The result is a holographic grating that can diffract light. Uniform illumination smears out the charge pattern and eventually erases the hologram, so photorefractive materials could be used for dynamic holography.

Until the past few years, all materials showing the photorefractive effect were inorganic crystals. Work going back to the mid-1960s (reviewed in ref. 2) has demonstrated that photorefractives could in prin-

ciple be used for optical processing, holographic, optical limiting, phase conjugation and storage applications. But their use has not become widespread, partly because of the difficulty and expense of growing and doping the required low-symmetry inorganic crystals.

Holographic optical storage is a current favourite³. A system based on writing and reading multiple holograms in the same volume of material might offer high storage density; and if parallel-array page composers (two-dimensional light modulators) were used to input a pattern of information and charge-coupled devices to read it out, high data transfer rates would be possible.

Polymers have several potential advantages over inorganic crystalline photorefractives, primarily because the dielectric constant is far lower: this reduces the 'screening' of charges by their neighbours, and thus leads to larger values of stored electric field for the same trapped charge density. The compositional flexibility and ease of sample preparation, doping, and processibility of polymers are also in their favour.

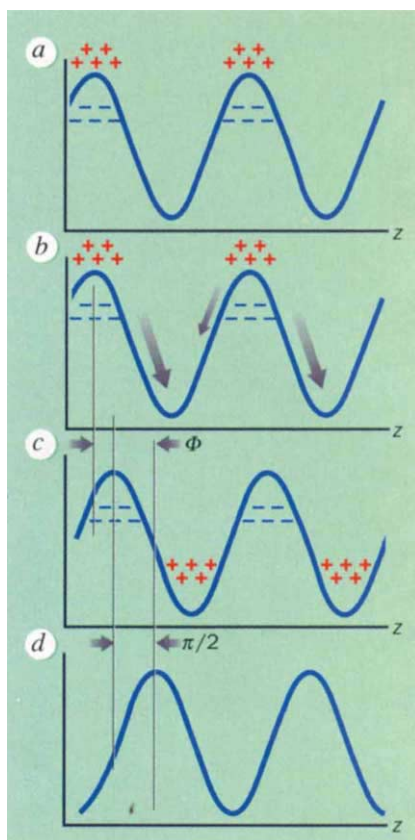
Optical nonlinearity is produced in polymeric materials by the presence of nonlinear optical chromophores, which

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The photorefractive effect

WHEN two light beams intersect to create an interference pattern in a photorefractive material, mobile charges are produced at the intensity peaks by optical absorption in charge-generating sites (a). If a uniform external field is applied, or if diffusion can occur, the positive and negative charges separate and drift apart to produce a 'grating' of electric charge throughout the volume of material (b, c). This produces an internal electric field that is also modulated at the periodicity of the original light intensity pattern, but is not in phase with it (d). If the material's optical index of refraction depends on the local electric field, as in the polymer developed by Meerholz *et al.* (page 497 of this issue), then a refractive index grating is a hologram which can diffract light. Its diffraction efficiency, defined as the ratio of intensity of the diffracted beam to that of a probing beam, depends on both the amplitude of modulation of the refractive index (Δn) and the thickness (L) of material, because the overall phase shift introduced into the beam is proportional to ΔnL .

In summary, if a material is to show the photorefractive effect it must contain a photoionizable charge generator and a charge-transporting medium, and its refractive index must depend on the local space-charge field. Deep charge-trapping sites, although not strictly necessary, are also desirable for long-



term storage of the space-charge field. These may be defect sites produced by the imperfect structure of the polymer, or the result of deliberate doping.

A key point is the phase difference Φ between the pattern of light intensity and the refractive index grating, which arises from the physical motion of charges in the material (they can move by as much as a few micrometres). This spatial non-locality allows for the asymmetric exchange of energy between two light beams in the material to yield optical gain. To see how it works, consider the two 'writing' beams that are interfering in the material to produce the index modulation. Each transmitted writing beam is accompanied by a diffracted beam in the same direction, produced by the other writing beam diffracting off the grating. As a result of the spatial phase shift, the transmitted and diffracted beams in one direction add constructively, yielding gain; the beams in the other direction add destructively, producing loss.

Beam coupling is at the root of many fascinating optical properties such as optical limiting, optical image amplification, or 'novelty filtering', in which a photorefractive material and simple optics are used to display only the features of an image that are changing with time. There are many other ways in which the refractive index of a material can be modified by an incident light beam — photochromism, thermorefractive and optical generation of electronic excited states, to name a few — but none produces the essential nonlocal effect and consequent phase shift. W. E. M.

are conjugated organic molecules with an asymmetric charge distribution produced, for example, by a donor substituent on one end of the molecule and an acceptor substituent on the other. To make the entire sample optically nonlinear, the nonlinear chromophores must be partially aligned (or poled) by an applied electric field in order to make the sample noncentrosymmetric⁴.

Charge-transporting polymers are already familiar as the organic photoconductors used in xerographic copying machines⁵. Generally, these contain a high concentration of 'transport agents', molecules which provide a network for charge to hop through the polymer. The sensitization of photoconductivity at a particular wavelength can be provided by a suitably chosen dye molecule in low concentration.

The first confirmed photorefractive polymer, developed in 1991 (ref. 6), was an optically nonlinear host polymer doped with a high concentration (30 weight per cent) of a charge-transporting hydrazone molecule. This material provided an important proof-of-principle that photorefractive polymers could be fabricated, but the maximum diffraction efficiency was

small (10^{-2} per cent in 350 μm thickness) and the grating growth times were large (minutes at 1 W cm^{-2} writing intensity). Additional polymeric photorefractives were quickly found, with substantial improvements in both diffraction efficiency and speed⁷. Recently we⁸ and independently workers at SUNY Buffalo⁹ took a different tack: start with the well-known photoconductive host polymer poly(*N*-vinyl carbazole), and dope it with a nonlinear optical chromophore in high concentration and a dash of sensitizer. Our approach proved fruitful, producing diffraction efficiencies greater than 1 per cent for the first time (in 125- μm -thick samples). More importantly, net gain was achieved in this polymer: the two-beam coupling gain coefficient was measured to be larger than the optical absorption coefficient (see box for explanation of the two-beam coupling process).

To understand the significance of the new result by Meerholz *et al.*, some simple facts about volume gratings are required. The diffraction efficiency of a volume hologram is proportional to the amplitude of the index of refraction modulation Δn as $[\sin(\pi \Delta n L / \lambda)]^2$, where L is the thickness of the sample and λ is the optical

wavelength. Thus, when $(\Delta n L)$ is small compared to λ , the diffraction efficiency grows proportional to $(\Delta n L)^2$. One might think that diffraction efficiencies could be made larger and larger by increasing the sample thickness, and (ignoring absorption) this is true up to a point for most inorganic materials where millimetre- or centimetre-thick samples can be fabricated (albeit with some difficulty). With the new photorefractive polymers, however, millimetre-thick samples are difficult to make at present, mostly because high electric fields are needed to align the nonlinear chromophores and to assist in charge motion.

When $(\Delta n L)$ becomes large enough, the sinusoidal function reaches unity (100 per cent diffraction efficiency) and then oscillates. Thus the actual diffraction efficiency never actually exceeds 100 per cent, but the index modulation (Δn) is large enough for 100 per cent efficiency to be attained with an even thinner sample. The new material¹ enters this regime and beyond, producing several oscillations in diffraction efficiency even with 105- μm -thick samples. (The maximum diffraction efficiency measured was only 86 per cent because of optical absorption and reflection.)

tion losses in the sample.) At the same time, the net gain coefficient describing the two-beam-coupling process was observed to reach values greater than 200 per centimetre, more than an order of magnitude larger than even our previous record-holding polymer. All this was achieved with subsecond response times at light intensities of 1 W cm^{-2} .

Although more experiments are needed to understand the new material fully, the greatly improved performance is probably due to three effects. First, the electric field used is near $100 \text{ V } \mu\text{m}^{-1}$, and the diffraction efficiency scales as the fourth power of the field in most materials. Second, a plasticizing agent is added to depress the glass transition temperature, so that the field can produce more alignment of the chromophores and hence more optical nonlinearity. Finally, Meerholz *et al.* suggest that the large response is partly due to an orientational enhancement mechanism¹⁰ not present in inorganic or crystalline organic photorefractives. This mechanism depends on the ability of the chromophores to orient in the total electric field at ambient temperatures, so that both the birefringence of the sample and the electro-optic coefficient are periodically modulated by the space-charge field. Although the nonlinear chromophore used does not have a larger hyperpolarizability than the one we were using last year, it is longer and more extended, and would thus be expected to have higher birefringence.

Despite the fact that photorefractive polymers were first identified less than four years ago, their index modulation and net gain are already superior to those of some of the well-known inorganic crystals, such as LiNbO_3 , strontium barium niobate and BaTiO_3 . Of course, much remains to be done before true practical application can occur, because other properties such as optical quality and grating storage lifetime have to be optimized. But the wide array of new materials and the leaps in performance over the past few years testify to their flexibility and promise. □

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How to make the gradient

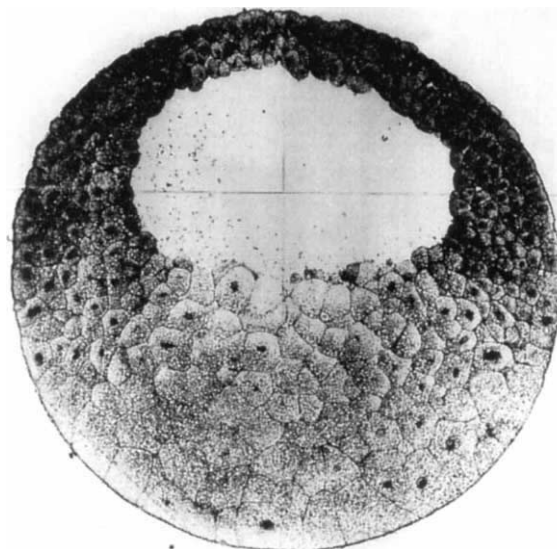
J. M. W. Slack

On page 487 of this issue¹, John Gurdon and colleagues describe how they have taken a fresh look at how morphogenetic gradients work in the early stages of animal development. The gradient is an idea of long standing. It goes back to the experiments of Child in the early years of the present century, which showed differences of regenerative capacity along the

source and that of *Xbra* further away, as would be expected if a concentration gradient of activin existed in the responding tissue. The range of the signal is at least $300 \mu\text{m}$ from the source, corresponding to about ten cell diameters given the size of cells in the late blastula. Gurdon *et al.* show that the zones of induced gene activity do not result from cell movement,

because strips of cells labelled with different fluorescent dyes do not become rearranged during the course of the experiment. Moreover, it seems that the activin itself probably diffuses across the tissue, rather than some substance released as a downstream effect, because the signal is able to cross a strip of non-responsive cells taken from a later embryo.

The availability of this model system raises a number of questions. First, the putative activin gradient has not actually been seen. An obvious extension to the work would be to make the concentration gradient visible by immunostaining, perhaps with the help of a *myc*-tag, in order to find out about its shape. We should like to know whether it is exponential, as predicted



Section through the *Xenopus* embryo at the developmental stage (blastula) used by Gurdon *et al.*¹ in their experiments. In normal development the mesoderm is induced in the ring of cells around the equator. (Reproduced from *The Early Development of Xenopus laevis* by P. Hausen and M. Riebesell, Springer, 1991.)

length of lower animals such as flatworms and hydroids². In the 1930s, the concept was extended by Huxley, de Beer and others to become a possible explanation for the specification of parts during embryonic development³. Then, in the 1960s, Wolpert rescued it from the obscurity into which it had fallen, and gave it a new lease of life as a potential mechanism underlying positional information⁴.

Using amphibian embryo material, Gurdon *et al.* have now studied a model morphogen gradient that promises to answer many of the questions still remaining about the behaviour and potentialities of embryonic signalling systems. The experiments involve the creation of an artificial signalling centre by the injection of activin messenger RNA into *Xenopus* early embryos, followed by a study of the spatial character of the responses using *in situ* hybridization to mRNAs produced by early mesodermal marker genes.

It is already known that these genes — *Xgoosecd* (*Xgscd*) and *Xbrachyury* (*Xbra*) — can be turned on respectively by high and low levels of activin. In the new work, the zone of *Xgscd* is found near the

by the localized source/dispersed sink model⁵, or linear, as predicted by the localized source/localized sink model⁶, or has some other form not predicted by an existing model.

Second, it would be interesting to know what pathways the activin actually moves through. Anyone who looks at electron micrographs of *Xenopus* blastulae must be

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impressed by the rather small amount of space between the cells in the marginal zone, so the cross-section available for diffusion must be very small indeed. It seems very unlikely that a protein such as activin could actually pass through the cells, so one wonders whether the cell surface or extracellular matrix embodies some special transport mechanism. If it does, then it is presumably shared by the non-responding cells used in Gurdon's experiments.

Third, there is the question of the sharpness of the responses. Although experiments involving cell reaggregation have shown sharp threshold responses⁷, it is now known that the initial activation of *Xbra* following activin treatment follows a smooth dose-response^{8,9} and after a few hours the boundary sharpens, both *in vivo* and in the present experiments. One way

of creating a sharp threshold is by a bistable circuit in which there is a positive feedback that can amplify the level of a substance once it is raised above the critical threshold¹⁰, and it has recently been shown¹¹ that there is a potential bistable loop in the early *Xenopus* embryo made up of the genes encoding the *Xbra* product and embryonic fibroblast growth factor. The operation of this circuit may perhaps be the mechanism that sharpens the initially smooth response.

In their paper, Gurdon *et al.* are at some pains to argue for the importance of activin itself in early *Xenopus* development. This is perhaps exaggerated, because there is very little activin protein in the egg and levels of new activin transcription remain very low before the neurula stage^{12,13}. Furthermore, inhibition of endogenous activin by over-

expression of follistatin produces essentially no phenotype¹⁴.

In my view, the significance of the present work is not so much that it tells us specifically about *Xenopus* development, but that it uses the versatile *Xenopus* embryo to create a system which can be used to study the general spatial properties of cellular signalling and response. This could be relevant not just to the problems of embryonic development, interesting though they are, but also to comparable situations in the adult organism such as tissue regeneration, the organization of epithelial proliferative units, or the interactions within the stem cell 'niches' of the bone marrow. □

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OBITUARY

Karl Popper (1902–1994)

WITH the death of Sir Karl Popper on 17 September, the scientific community has lost the philosopher who many of us feel illuminated, far more than any other, the way in which we work. His notions could be used with great advantage to evaluate whether a particular piece of research should or should not be regarded as scientific. Although many scientists have little interest in the philosophy of science, and some of those with such an interest are not too keen on Popper's teaching, to me his thoughts came as a flash of brilliant light. In this attitude I was proud to join Peter Medawar, Popper's most influential follower in the British scientific world.

Popper lived in the Vienna of the Positivists until his mid-thirties, then lectured in New Zealand. In 1945 the London School of Economics, to its lasting credit, brought him to England, where he remained. His seminal work in the philosophy of science, published in German in 1934 as *Die Logik der Forschung*, became available in English only in 1959, but its principal ideas were already well appreciated in the English-speaking countries by that time. The most immediate one is that science is demarcated from other endeavours (or should one say 'defined?') by the primacy of empirical disproof: only theories which can, at least in principle, be found to be wrong by experiment or observation are entitled to be called scientific.

This criterion is of great value in many fields of science. But Popper's contributions to the philosophy of science go far

beyond this hypothetico-deductive outlook. He was very clear that determinism makes little, if any, sense. Indeterminacy is to be welcomed, not regretted. If earlier philosophers had had Popper's insight it might have become clear long ago that Dalton's 'identical' constituents

sions of psychoanalysis and of Marxism was brilliant and effective. Indeed Popper's political philosophy, especially as displayed in his *The Open Society and its Enemies*, is most impressive for its pragmatism and humanity. It is a powerful call for democracy and a severe indictment of those who are so sure of their prescriptions that they are prepared to slaughter millions in their pursuit. A most accessible introduction to his thoughts is to be found in Bryan Magee's little paperback *Popper* (Fontana, 1973).

I must also speak of Karl Popper as a person. I had the pleasure of knowing him, and I particularly recall visits to his home in Penn when his kind, self-effacing wife Hennie was still alive. The atmosphere in that quiet house was so purely intellectual that nothing else seemed to exist. It was reminiscent of Hermann Hesse's *Glass Bead Game*. Indeed he often gave the impression of living in a world of his own, an impression reinforced by his deafness and his extreme objection to tobacco. Nonetheless, honours were showered on this retiring little man in his later life

— I like to think that he particularly enjoyed the recognition by the scientific establishment when he became a Fellow of the Royal Society in 1976.

A remarkable and most memorable personality is gone, but a life wholly filled with intellectual labours is made permanent by his many books. Hermann Bondi

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IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Popper — a remarkable and most memorable personality.

of matter necessitated a new kind of statistics, and similarly the thought that a maximum velocity for signalling put universal simultaneity in question might not have had to wait for Einstein. Popper's attitude to the Copenhagen interpretation of quantum theory has struck less of an echo, and he took some time to come to a positive view of the status of the theory of evolution.

His demolition of the scientific preten-

Lucinda Douglas-Menzies/Routledge

Night thoughts, dark sight

Charles H. Bennett

As a child I often lay awake wondering why I had been born as me rather than, say, my best friend. At other times I wondered whether unexposed photographic film has a colour, and whether it is the same as the colour of the exposed film one sees when a loaded camera is opened by mistake. Is it even meaningful to speak of colour in the absence of light? After a while I dismissed these questions as semantic puzzles, but at a meeting this summer* P. Kwiat, H. Weinfurter, T. Herzog and A. Zeilinger (Univ. Innsbruck) and M. Kaservich (Stanford Univ.) came closer than any before to inventing a way of seeing in total darkness.

In particular, they can determine whether an opaque object is present or absent in a given location, by an experiment involving only one photon, under conditions which virtually guarantee that the photon is neither absorbed nor scattered by the object. They described their method not as a way of examining unexposed film, but rather as a safe way to test for the presence of a hypothetical light-sensitive bomb, a bomb so sensitive that it would explode if struck by even one photon.

Before saying how it is done, we consider two simpler ways of using a photon to

A semi-reliable and semi-destructive measurement scheme was devised in 1993 by Avshalom C. Elitzur and Lev Vaidman of Tel Aviv University, who introduced the bomb to dramatize their idea (*Found. Phys.* **23**, 987; 1993).

In their scheme (Fig. 1), the photon is split between two beams of a Mach-Zehnder interferometer, and only one of the beams is allowed to pass through the location where the bomb might be. The one-photon Mach-Zehnder interferometer, which uses a half-silvered mirror to split the photon between two optical paths and then recombines the paths downstream at a second half-silvered mirror, is a textbook illustration of wave-particle duality. If no obstruction is present in either path, and if no attempt is made to determine which path the photon follows, the photon behaves like a wave traversing both paths simultaneously, and exhibits interference in the form of complementary oscillations in the detection

probabilities at the two detectors as the length of either path is varied. On the other hand, if a measurement is made to determine which path the photon took, no interference occurs, and the photon behaves like a particle, taking one of the paths at random. In Elitzur and Vaidman's interferometer the two paths are equal, so that if no bomb is present, the photon always appears at D1, and never at D2 (the detectors are assumed to be perfectly efficient).

Elitzur and Vaidman went on to note that if the bomb is present, three outcomes are possible. First, with probability $\frac{1}{2}$ the bomb explodes, proving that it was present; second, with probability $\frac{1}{4}$ the photon arrives at detector D2, proving that the bomb is present but not exploding it (the bomb must be there, they argue, to disturb the destructive interference, but it must not have absorbed the photon, as the photon arrived at the

detector); and finally, with probability $\frac{1}{4}$ the photon arrives at detector D1, yielding no definite conclusion about the bomb.

By repeating the experiment until one of the first two outcomes is obtained, one can determine definitely whether the bomb is (or was) present, at the cost of a $\frac{2}{3}$ chance of exploding it — a reliable but semi-destructive measurement.

The Innsbruck-Stanford group has now

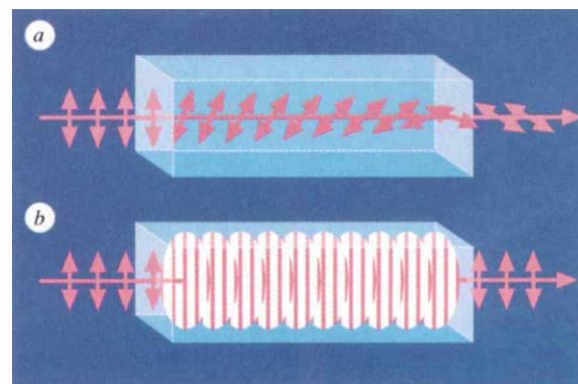


FIG. 2 Quantum watchdog effect. *a*, Undisturbed photon propagating through chiral medium undergoes a steady rotation of its polarization axis. *b*, Repeated interaction with vertical polarizing filters prevents this rotation, even though the photon has only a small chance of being absorbed.

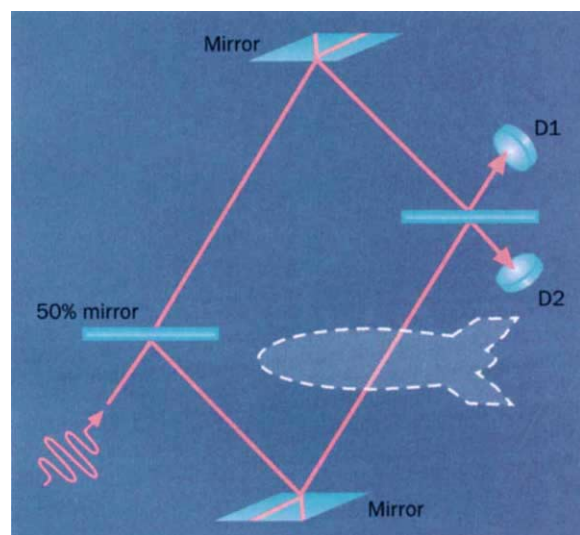


FIG. 1 Hypothetical one-photon interferometer to test for the possible presence of a light-sensitive bomb.

test for the presence of the bomb. The first is trivial and obvious — shine the photon at the place where the bomb might be. This finds the bomb at the cost of always exploding it if it is present. It may be described as a totally reliable but totally destructive measurement.

*Fundamental Problems in Quantum Theory, University of Maryland, 18–22 June 1994.

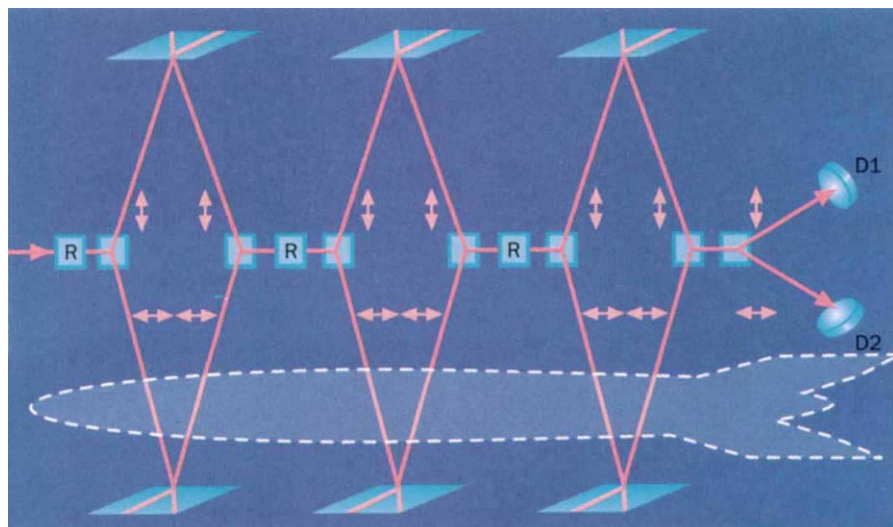


FIG. 3 Innsbruck-Stanford group's improved bomb-testing apparatus.

time it reaches the first filter, the photon's polarization is 9° off vertical. The filter forces the photon to choose between two outcomes: with probability $\cos^2 9^\circ \approx 0.976$ it is transmitted and becomes repolarized vertically; or with the complementary probability $\sin^2 9^\circ \approx 0.024$ it is absorbed by the filter. If unabsorbed, the photon continues through the second 10 cm of syrup, again having its polarization rotated by 9° ; the second filter again repolarizes it vertically with probability 0.976 or absorbs it with probability 0.024. Repeating these steps eight more times, one can see that two overall outcomes are possible: with probability $0.976^{10} \approx 0.78$ the photon is not absorbed by any of the filters, and emerges vertically polarized from the last one; with the complementary probability $1 - 0.976^{10} \approx 0.22$ the photon is absorbed along the way. In the limit of many equally spaced filters, the probability $1 - \lim_{n \rightarrow \infty} \cos^{2n}(90/n)$ of absorption goes to zero and the photon always emerges vertically polarized. Repeated interaction with the filters has prevented the syrup from rotating the photon's polarization: this is the watchdog effect.

If one regards the stack of n equally spaced filters as a single object, which might or might not be present in the syrup, the watchdog effect provides an unobtrusive way of testing for its presence: the photon will emerge vertically polarized if the stack is present, and horizontally polarized if it is absent, and only rarely will be absorbed.

The Innsbruck-Stanford scheme (Fig. 3) provides a way of using the watchdog effect to test for arbitrary opaque objects (such as a bomb) rather than special ones such as polarizing filters. A single vertical photon entering on the left passes through a small thickness of syrup, sufficient to rotate its polarization $90/n$ degrees, then is split by a polarizing beamsplitter into vertically and horizontally polarized beams. These beams are later recombined

by another polarizing beamsplitter. If no obstruction were present in the beam paths, and if both were of equal length, the original rotated polarization would be reconstituted exactly at the second beamsplitter. However, in the Innsbruck-Stanford scheme, one of the paths — the one containing the horizontal component — is sent through the suspected bomb location. Therefore the beam leaving the second polarizing beamsplitter is rotated $90/n$ degrees if the bomb is absent but remains vertically polarized if the bomb is present.

This process is repeated $n - 1$ more times, so that the photon emerges horizontally polarized if the bomb is absent but vertically polarized if the bomb is present (and has not exploded). A final polarizing beamsplitter and two detectors allow these two outcomes to be reliably distinguished. The explosion probability is $1 - \cos^{2n}(90/n)$, approaching zero for large n . Practical applications of the scheme remain to be developed; perhaps it could be used in airports, to look for bombs without exploding them and without harming even the most sensitive photographic materials carried by innocent passengers.

My other childhood question remains outside science, but it symbolizes a similarly vexing and metaphysical question in quantum mechanics: in any given trial of a probabilistic quantum experiment, why does one outcome occur and not another? Some physicists find comfort, others only further vexation, in the view that 'in reality' all outcomes occur in superposition, but our status as a subsystem of the Universe allows us to perceive only one of them, somewhat as our status as individuals causes each of us to perceive a unique self. □

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DAEDALUS

Maritime slime

THE maximum speed of a working ship gradually declines. Weeds, shellfish and other marine organisms steadily spread over its wetted surface, greatly increasing its drag. They adhere tenaciously, and are very hard to scrape off. The standard defence is antifouling paint, which releases poisonous organotin compounds. This poses ecological hazards, especially in inland waterways. Daedalus now has a greener solution.

He sees a ship's hull as a specialized marine habitat. He wants to cover it with a coating of organisms of very low drag that are so perfectly matched to this habitat that they colonize it to the exclusion of all rivals. Then their strong adhesion, rapid spread and self-renewal would no longer be exasperating, but beneficial. Instead of having to fight ecology, the marine engineer could welcome it cheerfully on board.

The green, scummy marine rock-algae seem ideal for the job. As many bruised beachcombers can testify, they are extremely slippery. Daedalus reckons that marine plants exude slime to reduce the drag of moving water, which might otherwise dislodge them in rough weather. This is the Toms effect, the reduction of liquid friction by low concentrations of a high-molecular-weight polymer. It has already been used to smooth the flow of water through fire hoses and reduce the drag of torpedoes.

So DREADCO's biologists are creating an algal paint for ships. One team is studying marine algae, seeking species that form the smoothest layers on a submerged surface. They hope to optimize a ship's bottom perfectly to the chosen algae by giving it a surface finish and chemical composition that welcome the algae but discourage their rivals. The algae should then displace alien weeds, shellfish and so on.

Another team is looking at seaweed slimes, and identifying the genes that produce them. When they have found the slimiest slime, they will insert multiple copies of its gene into the chosen algae. The result will be a tenacious, self-renewing, unbelievably slippery marine paint. Ship owners will dance a joyous hornpipe as their slimy vessels steam the world faster, longer, and with less maintenance.

Slippery algae will be happiest on translucent fibreglass hulls, through which light will leak to aid their photosynthesis. They may also find their slimy way into translucent plastic pipes inland, speeding the flow of industrial cooling water, outfall discharges and so on. They might even act as a biological filter and purifier.

David Jones

Is sea level rising or falling?

SIR — Sahagian *et al.*¹ estimated that the net effect of groundwater mining, wetland drainage, deforestation and water impoundment in reservoirs adds 0.54 mm per year to global sea-level rise (SLR). Our calculations, however, suggest that these processes may have acted to reduce sea-level rise by an amount comparable to that observed. Further, it is improbable that water diversions within internally draining basins (for example the Caspian

oxidation, shifting cultivation and long-term decay of wood, but not possible changes in runoff resulting from deforestation. Forest clearing may increase runoff in some cases and decrease it in others. One recent estimate³ suggests a net decrease in runoff from deforestation of 356 mm yr⁻¹. Given an annual deforestation rate of 15.4×10^{10} m² yr⁻¹ (ref. 4), the reduction of runoff may be approximately 55 km³ yr⁻¹, equivalent to -0.15 mm yr⁻¹ SLR.

A major reduction in SLR comes from sequestration of river flow behind dams (*R*). Sahagian *et al.*¹ significantly underestimate the volume of water impounded by dams^{5,6}. The largest reservoirs (> 3 million m³; ref. 7) have a total storage capacity of 3,991 km³. A more plausible value is approximately 5,000 km³ (ref. 8), equivalent to 14 mm SLR, at an average annual rate of -0.23 mm yr⁻¹ since the 1930s.

This storage capacity represents a lower bound of

SLR reduction from reservoirs. Additional reductions come from seepage and surface evaporation. Percolation beneath reservoirs recharges aquifers, ultimately increasing groundwater discharge to the sea, but this effect could be relatively small over short (100-yr) periods. If annual seepage losses due to deep percolation from reservoirs is about 5% of reservoir volume⁹, this amounts to 250 km³, averaging 0.69 mm yr⁻¹ withheld from SLR over the past 60 years.

Shiklomanov⁸ estimates an annual loss of 170 km³ by evaporation from reservoirs in 1990. A portion of the evaporated water is stored as vapour in the atmosphere, while the remainder returns to the surface cyclically as precipitation, a part of which flows to the sea. Although the fraction of evaporation withheld is uncertain, assuming it is only 10 per cent would reduce SLR by 0.047 mm yr⁻¹ ($170/360 \times 0.1$).

Similarly, losses of irrigation water (*I*) to deep seepage and evaporation lessen the anthropogenic input to SLR. In 1989, 232.8 million hectares were under irrigation¹⁰. Multiplying by the average annual per hectare water use of 11,400 m³ (ref. 11) gives a volume of 2,654 km³, very close to Shiklomanov's estimate⁸ of 2,680 km³ H₂O withdrawn in 1990.

Irrigation efficiency is a measure of the fraction of applied water that is used consumptively, namely that is taken up and evapotranspired by crops. If the consumptive loss of water in irrigation is 2,050

km³ yr⁻¹ (ref. 8), and if — as in the case of evaporation from reservoirs — 10% remains in the atmosphere as added vapour, then the amount withheld through evapotranspiration is 0.57 mm yr⁻¹.

The evaporation of 76% of irrigation water leaves a remaining 24% lost in seepage, only a small part of which replenishes aquifers. If, as in the case of reservoirs, only 5% of the water applied in irrigation ends up in deep percolation, the amount withheld is 0.37 mm yr⁻¹. These estimates are summarized in the table.

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Eco-friendly cups?

SIR — Hocking¹ has not compared like with like, as far as I can see. He compared the costs of production of reusable and disposable cups, but although he then added to the reusables the cost of reuse, he did not add to the disposables the cost of disposal.

If without disposal costs a ceramic or glass cup must be used about 40 times before the drinker uses less energy than drinking out of styrofoam or paper (and that's not counting those who reuse reusable cups several times without intervening washing), how many less times do we have to drink from reusables to make them equivalent to disposables once disposal costs are counted? Already, 40 drinks is perhaps only about one month's use, far less time than most of us manage to keep a cup.

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ANTHROPOGENIC CONTRIBUTIONS TO
SEA-LEVEL RISE OVER THE PAST 60 YEARS

Process	Sea-level rise (mm yr ⁻¹)
Groundwater mining*	0.39
Deforestation	
combustion, oxidation;	0.030
runoff	-0.15
Wetlands loss*	0.006
Reservoirs and dams	
storage	-0.23
infiltration	-0.69
evaporation	-0.047
Irrigation	
infiltration	-0.37
evapotranspiration	-0.57
Total	-1.63

*From ref. 1.

and Aral seas) will directly affect SLR.

The direct anthropogenic contribution to sea-level rise over a specified time period (SLR_a) is: SLR_a = (*G* + *D* + *W*) - (*R* + *I*), where *G* is SLR due to groundwater mining; *D* is SLR due to deforestation; *W* is SLR due to drainage of wetlands; *R* is SLR reduction due to impoundment in reservoirs; and *I* is SLR reduction due to irrigation. Strictly speaking, the terms are not entirely independent; for example, part of mined groundwater goes into irrigation. Here we estimate values for *D*, *R* and *I*, as discussed below.

The net atmospheric carbon release from tropical deforestation (*D*) ranges from 1.1 to 3.6×10^{15} g C yr⁻¹ (ref. 2), with a likely mean of 1.7×10^{15} g C yr⁻¹ (R. A. Houghton, personal communication). Complete combustion of vegetative cellulose produces CO₂ and H₂O: 6 O₂ + C₆H₁₀O₅ → 6 CO₂ + 5 H₂O. The mass of carbon burned each year is equivalent to 2.125 km³ H₂O, or only 0.006 mm yr⁻¹ SLR, even if all of the water flowed to the sea (1.7×10^{15} g C yr⁻¹ × 1.25/1.0 g cm⁻³/360 km³ 1 mm⁻¹ SLR).

The water content of vegetation cleared each year is obtained using a dry-to-wet biomass ratio of 0.25 (ref. 1). Conversion of the carbon to water yields 8.5×10^{15} g H₂O, equivalent to a 0.024 mm yr⁻¹ SLR, if all that water were added to the ocean (1.7×10^{15} g C × 4 × 1.25/1.0 g cm⁻³/1 mm yr⁻¹ SLR).

These figures² include burning, topsoil

HOCKING REPLIES — I did, in fact, consider disposal costs — see, for example, ref. 4 in ref. 1 (cited here as ref. 2). Van Eijk *et al.* have considered the bulkier packaging used for shipping and cleaning agents for ceramic cups, plus the larger landfill volume required by a reusable cup on eventual discard². From this information, they obtained break-even values for the landfill volume consumed by porcelain/polystyrene and porcelain/paper cup pairs of 125 and 99, respectively. In other words, one could use once and discard 125 polystyrene cups before the landfill volume consumed would equal the landfill volume consumed by 125 uses of a porcelain cup. Fewer uses than these gave lower landfill volumes per use for the disposable cups than for the porcelain cup. At more than 125 uses of the porcelain cup the landfill volume occupied per use gradually dropped relative to the disposable options so that at 3,000 uses this required a total of 2.4 litres whereas the polystyrene and paper options required 9.1 and 11 litres, respectively.

Reuse of cups, whether reusable or not, expends more resources than recycling. This factor, plus aesthetics, keeps reusable cups dominant in beverage service. Polystyrene (or other plastics) and paper can be materially useful in either recycling or energy recovery programmes, the next levels of resource use. Glass can be recycled, saving at least landfill space, as the energy required to transport glass locally and remelt it is scarcely less than that of transporting and melting the raw materials. But ceramic cups that have reached the end of their useful life have no recycle or energy-recovery attributes.

Some readers have written to me direct to question the energy costs of initial cup delivery, among other factors. I did include delivery energy in my calculations¹. Non-energetic costs such as destroyed natural habitat are difficult to quantify for diverse technologies^{3,4}. But the combined habitat impacts of a large-scale china clay processing facility and chinaware firing line are at least of a similar order to those of a pulp mill or an oil-refinery complex. The air pollution impact of the porcelain option is much higher than paper or polystyrene at a few uses². At 3,000 uses, this impact per use drops to about the same for porcelain and polystyrene and a slightly higher impact value for paper. Paper and polystyrene produce only about one-third of the water pollution impact of porcelain per use because of the need to wash reusable cups.

These details from our own and other studies confirm that there are situations,

when only a low number of cup-use cycles can be anticipated, where use of disposable cups could be more efficient and less harmful to the environment than use of any of the reusable cups. (The complete version of the calculations used in ref. 1 is available in ref. 5, which should be published in December.)

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Avalanche survival chances

SIR — Falk *et al.*¹ in their interesting Scientific Correspondence demonstrated the high risk of death due to burial by avalanches, and the importance of avoiding them. I cannot fully agree, however, with their conclusion that the depth of burial has no direct influence on survival chances.

I have analysed mountain sports accidents in Austria since 1986. In cases of

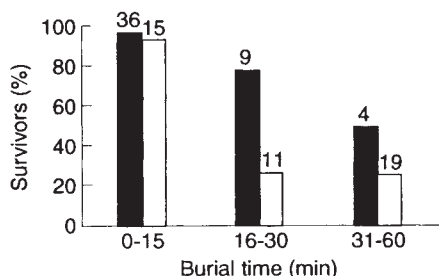


FIG. 1 Black bars, percentage of survivors buried at, or at depths less than, the overall median burial depth (50 cm); white bars, those buried deeper than this. Figures above the bars indicate the number of buried skiers within each subgroup.

burial by avalanches, I recorded the rescue outcome, burial time and depth of burial. In a period of 7 years (1986–92), 774 people were caught in avalanches; of these, 380 were buried completely (the entire body covered by snow) and 148 were killed. Of the totally buried skiers, only 94 were known reliably to have been buried not more than 1 hour; of these, 28 (30 per cent) were dead on extrication.

The percentage of survivors in three pre-defined time periods are presented for two subgroups which represent those buried either deeper than, or at or less than, the overall median burial depth (50 cm) (Fig. 1). Burial depths were 62 ± 43 cm (mean $\pm$ s.d.) up to a burial time of 15 min, 104 ± 61 cm at a burial time of 16–30 min, and 115 ± 42 cm at a burial time of 31–60 min. For those buried for 15 min or more, people buried less deeply had a greater chance of survival than those

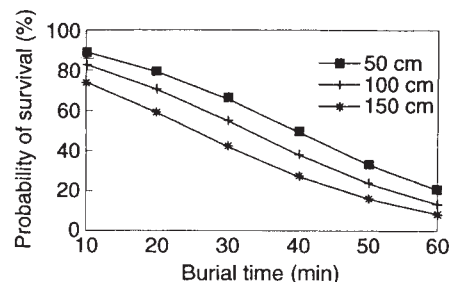


FIG. 2 Time- and depth-dependent probability of survival based on the result of logistic regression.

buried more deeply.

The question arises whether the reduced survival chance at greater depth is the result of a longer extrication time, or whether an additional direct effect of burial depth exists. Analysing the data, I was able to demonstrate a time-dependent and time-independent effect of burial depth on the chance of survival. A correlation exists between burial time and burial depth ($r = 0.5$, $P < 0.001$), primarily due to the longer extrication time (time-dependent effect). I tested the direct influence of depth of burial on survival by applying stepwise logistic regression to rescue outcome (dependent variable) in relationship to burial time and depth (independent variables). Burial time entered the model at step 1 and depth of burial at step 2, and its presence effected a significant improvement compared with step 1 (improvement $\chi^2 = 3.76$, $P = 0.05$). The resulting model for the probability of survival is:

$$\frac{e^{3.232 - 0.06796t_b - 0.01034d_b}}{1 + e^{3.232 - 0.06796t_b - 0.01034d_b}}$$

where t_b is burial time and d_b is burial depth. The probability of survival at various burial depths based on the result of logistic regression is presented in Fig. 2. The time-independent effect of burial depth on the chance of survival is probably brought about by increased traumatization and thorax compression at a greater depth. This assumption is also supported by Stalsberg *et al.*², who showed that the immediate cause of death of avalanche victims in most cases was general body compression with acute respiratory and circulatory failure. Hence, when an avalanche begins, all measures helping to keep the skier on the surface increase the chance of survival.

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Net profit or net loss?

J. H. S. Blaxter

Scaling Fisheries: The Science of Measuring the Effects of Fishing, 1855–1955. By Tim D. Smith. Cambridge University Press: 1994. Pp. 392. £50, \$74.95.

Net Loss: Fish, Jobs and the Marine Environment. By Peter Weber. Worldwatch Institute: 1994. Pp. 76. \$5 (pbk).

On the Sex of Fish and the Gender of Scientists: A Collection of Essays on Fisheries Science. By Daniel Pauly. Chapman and Hall: 1994. Pp. 250. £35.

THE most recently available statistics from the United Nation's Food and Agriculture Organization give a worldwide fish catch of 97 million tonnes in 1991, of which 82 million tonnes were taken in the sea. In fact, the world fish catch grew at an annual rate of nearly 6 per cent after the Second World War but is now increasing at only about 2 per cent. These facts may seem

are well described in *Scaling Fisheries*. In the second half of the nineteenth century, high-seas fishing became increasingly important (and apparently even threatening to the stocks) as the use of steam to drive vessels and winches bolstered the power of the fleet. That high-seas fish stocks were a resource shared among nations was abundantly clear, with international collaboration thriving from the start. As fishery science grew, new techniques were quickly developed. Two of the most important were the determination of the age structure of stocks and therefore the ability to determine growth, and tagging of fish to investigate migrations. Later, the invention of the echo-sounder made it possible to follow concentrations of fish from day to day and, much later (beyond the historical scope of the book), to determine their biomass.

Patrick Friel/Rex Features

A sophisticated younger reader of the book may be amazed at the apparent naivety of the first fishery biologists. They seemed to have had some pretty cock-eyed ideas, which could have been easily dispelled

by modern equipment, techniques and insights. But bear in mind, to give an example, that in the late 1800s fish eggs could not be identified to species, so the technique of biomass estimation from a census of early life-history stages was not an option.

Methods of management have swung in and out of favour. One of the most notorious was the idea of planting large numbers of eggs or newly hatched fish larvae in the sea to enhance stocks. Some countries maintained this expensive practice long after it was shown to be useless. Yet today the idea has been resuscitated, but this time with the planting out of much older stages, which are better able to fend for themselves.

One of the most severe problems for both fishery scientists and managers is the large year-to-year variation in recruitment to fish stocks. Remedial measures are

taken against a very 'noisy' background, often making it difficult to determine whether a measure has been successful or not. To obtain a maximum sustainable yield from a stock — obviously the best long-term goal — it was realized early on that young fish must be allowed to enter the stock and not be caught by the nets. Much early management depended on increasing the mesh size of the nets to allow the small young fish to pass through and on establishing minimum sizes for landing; these practices are still of great importance. More recently, again outside the scope of the book, total allowable catches have been introduced so that the catch of a particular stock may be divided among the appropriate fishery nations. Even this package of measures is failing to allow over-exploited stocks to recover and it has been realized for some time that fishing pressure must be reduced by restricted entry or reduction of the number of fishing vessels. The most dramatic demonstrations of the effects of reduced fishing were the high takes of fish in European waters after the First and Second World Wars.

Tim Smith's book makes interesting reading for scientists and historians. Readers without specialized knowledge will understand it if they read it from the beginning. The style would have been more coherent if there had been fewer digressions and minutiae about the early fishery scientists themselves. Perhaps these were included to lighten the rather heavy main material, but they succeed only in deflecting readers. Another irritation is the constant jumping from one decade to another and back again, making it difficult to follow a theme. More regrettably, the author cuts off the historical account in 1955, preventing one from relating the herculean efforts of the earlier fishery scientists to more recent developments.

In *Net Loss*, Peter Weber takes up the tale 40 years later with a useful booklet on the present dangers to our fisheries and fishermen, not only from over-exploitation but also from pollution; he emphasizes the over-capacity of the fleets. Daniel Pauly's curiously misnamed book is a collection of essays on fishing with an emphasis on the tropics and developing countries. As might be expected, the problems of recruitment and over-exploitation are prominent. Its aim of getting away from the "relentless parochialism of the average published paper" succeeds, but it should never be forgotten that these papers are the bread-and-butter of the fishery scientist. So speaks the editor of a journal of marine science! □

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Caught in the act — herring fishing.

surprising given the constant and very public concern of politicians, managers and scientists about the poor health of fish stocks, with nearly all of the fish caught through hunting rather than raised in captivity.

A closer analysis shows a fluid and impermanent structure to the aggregate catch; over the years, some stocks have disappeared, others have been over-exploited and allowed to recover, still others have recovered of their own accord. Most importantly, new species have been exploited. In the 1960s and early 1970s a clupeoid (the anchoveta) dominated the catch in terms of weight, if not value. Today the most important species is a gadoid, the Alaska pollack, which has contributed 5–6 million tonnes annually over the past decade.

The alarms and concerns of scientists from the earliest days of fishery science

Mining the seams of our Solar System

Joseph A. Burns

Resources of Near-Earth Space. Edited by J. Lewis, M. S. Matthews and M. L. Guerrieri. University of Arizona Press: 1993. Pp. 977. \$85.

In the decade before 1492, when Columbus was shuttling across the Iberian peninsula seeking royal sponsors for his so-called "Enterprise of the Indies", the Genoan sea captain read three books over and over again. These volumes were presumably called upon by Columbus in discussion with various sovereigns to buttress his claim that Asia, with its kingdoms of astonishing riches, lay only slightly west of Europe. The best known work was Marco Polo's *Travels* which incorrectly reported a vast eastward extent to China and the presence of a fabulously rich Japan supposedly some 2,500 km further east off the Chinese coast. Another was a world geography written 75 years earlier by a French theologian-astrologer who conveniently maintained that the Western Ocean was quite narrow. A third was a compendium of enticing facts about China

prepared in 1477 by Pope Pius II; here the East was portrayed as a land of great wealth inhabited by bizarre beasts and peculiar peoples. Of course, today we know that the information in these ancient books, although among the most accurate of its day, was flawed. Nonetheless, the volumes made intriguing reading and they accomplished Columbus's purpose.

Resources of Near-Earth Space may play a role similar to Columbus's travelling library for the possible future exploration of Earth's celestial neighbours — the Moon, nearby asteroids, Mars and its two tiny satellites. This book contains brief, up-to-date descriptions of the target bodies and discusses how the materials on them might be exploited. Although one might expect space-age hucksterism on such a topic, the book does relatively little marketing. Only the introductory chapter, written by one of the editors, John Lewis, and two other space scientists, extols the economic potential of nonterrestrial resources, stating that these objects are "rich in materials of great potential value to humanity" that "in the long term . . . may be of great importance here on Earth". But even this chapter ends with a note of caution: although "we cannot yet answer [whether such ventures are economically viable] . . . it would be irresponsible not to seek the answers diligently".

About a third of the book's 30 or so chapters concern the physical and chemical properties of Earth's nearest neighbours, as understood largely from telescopic observations. These articles, written by established planetary scientists of excellent reputation, focus especially on various aspects of Apollo/Amor asteroids and Mars; by contrast, only single chapters are devoted to the Moon, short-period comets and the Martian satellites. In most cases, after reviewing current knowledge, the authors of these 'science' chapters attempt to interpret the known characteristics in terms of potential resources.

Much of the rest of the book, prepared by a refreshing combination of engineers, industrial technologists and space scientists, describes how these resources might be mined, extracted and processed in the hostile, low-gravity environment of space. For example, there are six chapters concerning the production of oxygen on the Moon,

and additional ones about the processing of lunar basalts as well as refractory and non-volatile materials. Several other studies describe possible volatile sources on the Moon, carbonaceous asteroids and Mars. And there are even discussions of how Martian resources might be called upon to provide life support and fuels for return voyages. These presentations are a mixed bag: some provocative, others trivial extensions of mundane terrestrial technology.

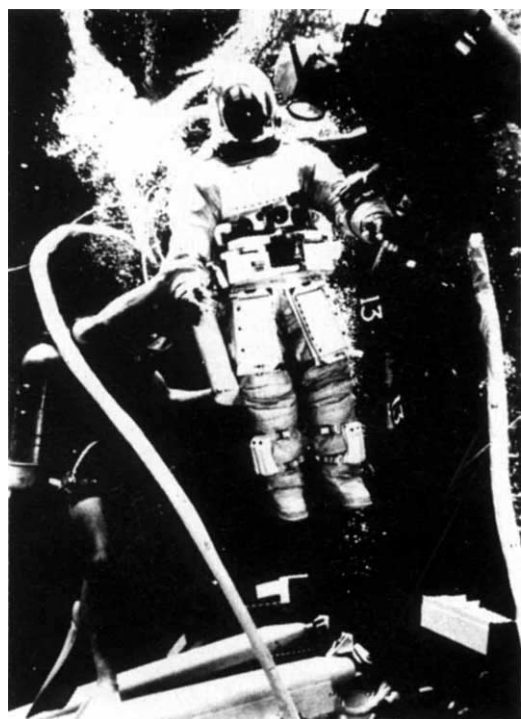
Only a few chapters present mission profiles, or address the logistics of transporting machines and necessary supplies to process the extraterrestrial materials and then to return the finished products to market on Earth. Even less time is spent analysing the pivotal question of whether today's (or tomorrow's) launch costs could make the exploitation of space profitable sometime in the foreseeable future.

Broadening its purview, the book ends with J. Pollack and C. Sagan's fairly general questioning of whether Mars or Venus could be made habitable through some grand work of planetary engineering; these authors conclude that the task is too great and, in any case, is questionable on ethical grounds. Instead they argue that humans, before attempting to improve the habitability of other worlds, should first safeguard Earth by countering global warming through a programme of extensive reforestation.

Resources of Near-Earth Space is principally valuable because it provides the first opportunity for practising space scientists to appreciate what knowledge futurists will need, while informing aerospace engineers of just how limited our current knowledge is about even Earth's nearest neighbours. The editors intend the volume to guide industrial and governmental project managers who are starting to consider the feasibility of using nonterrestrial materials. This volume certainly will not be the last word on the topic but it should generate considerable discussion among thoughtful futurists. Will the book be carried by contemporary Columboes as they go to request government support for their exploitation of some far-off land? I wonder. □

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■ Also recently published is *Preservation of Near-Earth Space for Future Generations*, edited by John A. Simpson. The book, which is based on a symposium held to mark the hundredth anniversary of the University of Chicago in June 1992, covers the technical aspects and the economic, legal and international issues surrounding the future uses of space. Cambridge University Press, £50, \$79.95.



BUBBLE chamber — a Skylab astronaut, assisted by scuba divers, in a "Neutral Buoyancy Simulator" test. Taken from *History of Rocketry and Astronautics, AAS History Series, Vol. 11*, symposia edited by Roger D. Launius. Published by American Astronautical Society, \$60 (hbk); \$40 (pbk).

A woman on the verge

Julia Neuberger

I am Roe: My Life, *Roe v. Wade*, and Freedom of Choice. By Norma McCorvey with Andy Meister. HarperCollins: 1994. Pp. 216. \$23.

THERE is among some of those opposed to abortion a view that runs along these lines: "Abortion is wrong. It should also not be necessary, since any woman can avoid pregnancy. A woman's incompetence should not entitle her to kill her unborn child." Clearly, this is not the view of those in the Roman Catholic Church and others who object to birth control. But

lesbian in the late 1980s. But in the days when she got pregnant and allowed her children to be adopted, including by her mother who deceived her into forsaking rights to her daughter, she was confused, angry, depressed, at one stage suicidal, and deeply disadvantaged. To say to such a woman that she simply should have been more careful is nonsense. To say to her now that girls should be taught about contraception would leave her unmoved. Her experience is that many girls will never get the chance to learn in the face of pressure from the moral majority in the United States, and increasingly in the United Kingdom, against any sex education that is explicit and protective.

McCorvey has felt some of the wrath of the anti-abortionists, who have attempted to shoot her and strewn baby clothes over her lawn. She has also experienced a difficult relationship with the young female lawyers who fought *Roe v. Wade*, discovering that the case itself, which lasted almost three years, meant it was too late for her to have an abortion. She believes, in the light of her experience, that it is a woman's right to choose.

Her story is deeply moving, yet there has to be an intellectual argument as well. She provides a brief history of the changing view of the Roman Catholic Church. Before 1869, the Church sanctioned termination of pregnancies up to day 40; until this age the fetus was thought to be unformed. But since then, ensoulment is considered to happen at conception, so abortion at any stage is immoral.

Nor is this view likely to change. The distinguished legal philosopher Ronald Dworkin, in *Life's Dominion*, published last year (HarperCollins, 1993), argues that a compromise on abortion is possible if people think about issues differently. He suggests that the whole debate is based on an intellectual confusion capable of being identified, analysed and dispelled, and adduces a "derivative" objection to abortion, based on the theory that fetuses, like grown human beings, have rights, including the right not to be killed. The individual who accepts this argument believes that governments have a duty to protect these rights.

But he also adduces a "detached" objection. Not based on rights or interests, this theory relies on the premise that abortion is wrong in principle because it "disregards and insults the intrinsic value, the sacred character, of any stage or form of human life". The person who holds this view believes that governments have a detached responsibility for protecting the intrinsic value of human life. He argues that most people hold the latter view, regarding it as "a bad thing, a kind of cosmic shame" when human life at any stage is extinguished. It is then perfectly possible for them to believe that abortion is morally wrong yet feel that the decision

must be left to the pregnant woman, as the person with the greatest stake in it.

It would be good to read this argument in *I am Roe*. McCorvey is not someone who will argue intellectually, but her coauthor and fellow campaigners might. McCorvey had made a strong case from experience for leaving the decision to have an abortion to the woman concerned. Her colleagues, with her, now need to add some of the intellectual and moral bite behind the experiential justification. □

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Physics in transition

Brian Pippard

Order, Chaos, Order. By Philip Stehle. Oxford University Press: 1994. Pp. 322. £44, \$59.95 (hbk); £29.95, \$22.50 (pbk).

THE first quarter of the twentieth century saw a comprehensive transformation of physical ideas. The elaborate structure built on Newton's dynamics and Maxwell's electromagnetism had already begun to show flaws when, within a few years, the discovery of X-rays, the electron and the quantum stimulated a radical rethinking that eventually led to the invention in 1925 of quantum mechanics.

There is rarely time to tell students what happened in this period, but historians of science have rightly found it fascinating. Some great innovators, such as Einstein, Rutherford and Bohr, have had their lives and works examined minutely, while the development of leading strands of thought has been mapped in detail. Scholarship at this level makes tough reading, but shows how little most of us appreciate how the revolution was achieved. Philip Stehle has successfully condensed the most important aspects into a single volume that can be read by anyone with a modest competence in physics. It is not a 'popular' book — the lives and loves of the principal actors are left alone — but it is one that deserves to be read by science students and their seniors. The disconcerting parade of mathematics has been largely avoided in the main text, but at the end of sections there are amplifications, clearly set apart, in which more mathematical detail is presented. There is no doubt that this is a serious exposition of an intellectually enthralling tale.

In his analysis of the order, Stehle deals lightly with the accumulation of problems that menaced and eventually destroyed Maxwell's ether — these have been tack-

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Norma McCorvey — abortion rights activist.

many passionate anti-abortion campaigners are not opposed to contraception. For them, life begins at conception, which is what renders abortion immoral.

These people should read this autobiographical story by Norma McCorvey, alias Jane Roe, the plaintiff in *Roe v. Wade*, the landmark Supreme Court case that legalized abortion in the United States in 1973. McCorvey was a woman who never had a chance in life. Her relationship with her mother was appalling from early on. She was never told the facts of life, and when she was first pregnant did not realize it. Her relationships with men were brief, often violent, and the pregnancies were resented.

Now she is a seasoned campaigner, who 'came out' both as Jane Roe and as a

led in depth by others and are very much a matter for specialists. But he illuminates the conceptions that brought Fitzgerald, Lorentz and Poincaré to the brink of relativity and to the taking-off point of Einstein's dramatic leap. The mathematical framework had been supplied by his forerunners, and there were others keen to follow where Einstein led. Not so in the matter of quantum theory. The arguments that convinced him of the need to think of radiation as discrete quanta carried no conviction in the minds of others; even Planck and his colleagues, eight years later, had to ask that this error of Einstein's should not be held too much against him. This is the story that began with black-body radiation and which, with the "allergic reactions to h ", is excellently told, and makes clear how much of accepted physical truth had to be replaced. Once one appreciates Einstein's extraordinary insight and courage, it is clear why quantum theory was as important to him as relativity, and why he did not let go of it even while his energy was concentrated on the General Theory.

Nearly half the book describes the evolution of atomic models, with Rutherford and Bohr as the leading spirits, of course, but with many others as well. The struggles to find how to apply quantum theory, and the limitations of the resulting planar hydrogen atom, are of small concern to modern physicists, as is the disheartening recognition of the old quantum theory's impotence in the face of the helium atom. Yet these struggles should not be forgotten, for they show how a heroic breed undertook a nearly impossible task and yet established a foundation for the later quantum mechanics. The great breakthrough by de Broglie, Heisenberg and Schrödinger marks the point at which a new order emerges, and the purpose of the book is achieved.

I have only one and very minor criticism of this splendid work, and that is its title. It is not that the word 'chaos' has a new meaning for modern readers, but that I doubt whether the period described was seen by the participants as chaotic, in their sense. To those excited by the prospects revealed by the electron and by radioactivity, there was no lack of stimulating experimental projects, and the mental agonies of the theorists (who were few in number) were surely recognized, even by themselves, as the sort of thing that anyone must expect in searching the fundamental mysteries. If today's problems of high-energy physics should suddenly be resolved, our own times may also come to be seen as chaotic, but I doubt if many of our leading physicists would accept this interpretation. □

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Protocols and conventions

Andrew Jordan

The Greening of Machiavelli: The Evolution of International Environmental Politics. By Tony Brenton. *Earthscan/Royal Institute of International Affairs*: 1994. Pp. 282. £29.95, \$54.95 (hbk); £14.95, \$24.95 (pbk).

ONE of the defining motifs of modern environmentalism has been the steady internationalization of environmental politics and policy-making. This has, in part, been propelled by the recognition that a globalized world economy is creating social and environmental upheaval that is cumulative, sometimes uncertain and often transnational. Prognostications of impending global environmental doom have, in turn, inspired broad social movements and myriad pressure groups, some of which now link up across countries and continents to harry governments and industry for 'greener' growth. In a political world divided into separate nation states, governments have normally responded to this pressure by negotiating international legal agreements (such as conventions and protocols) and creating international institutions (to lend money, regulate trade and settle disputes).

But just how successful have these responses been and, more importantly, are they, in their current form, capable of responding to future threats of global environmental degradation and social upheaval? The general conclusion of this book, a broad historical review of international environmental issues since the late 1960s, is that they are, but that cautious reforms could nonetheless be made to facilitate global sustainability. This judgement is sufficiently sanguine to place Tony Brenton firmly in the so-called 'light green' camp. In the opening chapter, for example, he declares that he is a "moderate" and an "unreconstructed anthropocentrist"; at heart he remains unconvinced that "we yet face an environmental crisis which will overwhelm us unless we make very rapid and dramatic changes to our lifestyle and aspirations".

In the chapters that follow, Brenton provides a fairly standard conspectus of the origins of modern environmentalism, the various responses of developed and developing countries to 'green pressure' (both domestically and internationally) and the more recent emergence of the so-called 'sustainable development' paradigm. For some issues there have been what Brenton terms "level one" responses (rhetoric, nonbinding agreements and little concrete action) whereas for others there are binding treaties, and purposive

action that has led to substantial improvements in environmental quality ("level three" response). In a number of more detailed studies of individual treaties, he uses this typology in an attempt to explain how and why some environmental problems have been 'solved' while others have not.

Sensibly, Brenton chooses to frame his analysis with chapters on the two high-tide marks of modern environmentalism: the United Nations conferences at Stockholm in 1972 and at Rio de Janeiro in 1992. In an engaging comparison of the two, he notes how each was characterized by the now familiar and seemingly intractable disputes between the North and South over priorities, burden-sharing, financing and target-setting. In four somewhat less original chapters, he discusses the political background to the Earth Summit in Rio, and assesses the prospects for future international action on climate change and biodiversity.

Throughout, Brenton gives short shrift to those 'deep' greens who warn of impending 'limits' to growth (they are "so remote", he contends, "as not to be a sensible concern for public policy") and who advocate a 'coercive' approach to environmental protection. Here Brenton is on highly contested ground. Although it is certainly true that some environmentalists have tended to downgrade issues of social justice, it is equally apparent that sustainable development is not synonymous with unlimited economic growth.

In his final chapter, Brenton argues strongly for appropriately scaled modifications to, rather than a radical restructuring of, the existing systems of free trade, international governance and financing. For Brenton, sustainable development will not emerge from "dense webs of regulation" or the "old 'command-and-control' style of environmental thinking", but only when there is more democracy, greater economic prosperity and a market that works for, rather than against, the environment.

Overall, this is a book that aims to identify and explain recurrent themes and patterns in modern environmental history by condensing large amounts of existing material into a relatively small space. As such, it will no doubt be of most use to those seeking a pithy exposition of the salient trends in contemporary international environmental politics. It does not, however, cover much new ground and, because the analysis is undertaken at a relatively high level of aggregation, it is unlikely to provide much for the cognoscenti. □

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Activin signalling and response to a morphogen gradient

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Using combinations of amphibian embryo tissues, it is shown that the selection of genes expressed by a cell is determined by its distance from a source of activin, a peptide growth factor contained in vegetal cells and able to induce other cells to form mesoderm. This long-range signal spreads over at least 10 cell diameters in a few hours. It does so by passive diffusion, because it can by-pass cells that do not themselves respond to the signal nor synthesize protein. These results provide direct support for the operation of a morphogen concentration gradient in vertebrate development.

MUCH current work is directed towards identifying molecules involved in embryonic induction and to describing their developmental effects. It is thought that some kinds of inducer molecules, acting as morphogens, are synthesized and emitted by cells in one region of an embryo and become diluted in concentration as they spread to cells distant from this point. A favoured hypothesis is that the type of gene activation and cell differentiation undergone by responding cells is related to the concentration of inducer that they receive^{1,2}.

The best evidence that this mechanism operates in vertebrate embryos comes from the important work of Green and Smith^{3,4}. They exposed dissociated *Xenopus* blastula cells to different concentrations of an inducer, namely activin, and found that the type of genes expressed in these cells is related to the concentration of inducer used. It has yet to be shown, however, that a similar effect can take place in normal solid tissue rather than in dissociated cells. Furthermore there is a clear need to test the morphogen concept directly by asking whether the kind of response that cells of a normal tissue make to a signal is determined by inducer concentration and therefore by their position with respect to the source of the morphogen.

Here we describe experiments in which we show (1) that an embryonic signalling process can spread for at least 300 μm through solid tissue; (2) that the selection of genes activated is related to the distance of cells from a source of signal, and presumably therefore to the concentration of a signalling molecule; and (3) that the signal is transmitted by passive diffusion rather than by active cell-by-cell amplification. Our results therefore provide direct support for the fundamental concept that selective gene activation takes place in response to morphogen concentration in normal embryonic tissue of a vertebrate.

Inducer concentration and response location

To ask whether there is a relationship between the strength of an inducer and the distance from its source at which particular genes are activated, we have used the following experimental system. A Nieuwkoop conjugate is constructed in which the vegetal hemisphere of a blastula is placed in contact with a blastula animal cap containing the fluorescent intracellular marker rhodamine lysinated dextran (RLDx). The vegetal hemisphere is taken from an embryo which had been injected at its two-cell stage with varying amounts of activin messenger RNA synthesized *in vitro* (Fig. 1a). The conjugates are cultured until the early gastrula stage, when they are fixed, sectioned and hybridized *in situ* for expression of the pan-mesodermal marker *Xbrachyury* (*Xbra*) (ref. 5). In normal embryos at the mid-gastrula stage, *Xbra* mRNA is seen to be localized in an internal ring in the

marginal or equatorial zone (Fig. 2e). Figure 2a–c shows that increasing amounts of activin mRNA in vegetal cells cause *Xbra* RNA to appear in animal cells located progressively further away from the activin source. The results of 25 analyses are summarized in Table 1. Many of the *Xbra*-expressing cells seen in Fig. 2a–c subsequently progress to muscle, as shown by their acquisition of strong nuclear staining for MyoD, a marker of muscle progenitor cells (Fig. 2d), and when cultured further express terminal muscle marker genes (not shown). We conclude that there is a direct relationship between the amount of inducer mRNA in vegetal cells and the distance from them at which mesoderm genes are expressed.

We have asked whether this distance effect of activin mRNA can be generated only by those cells that are natural sources of mesoderm-inducing signals. The injected activin mRNA might stimulate the production of some other endogenous inducer in these cells. If, on the other hand, the effect is related directly to activin itself, we might expect it to be generated by cells that are not themselves normal mesoderm inducers, for example by animal pole cells injected with activin mRNA (Fig. 1b). This is indeed the result obtained when activin-containing animal caps are conjugated to other non-injected animal caps (Table 1; see also Figs 3e and 4h). We conclude that the amount of activin mRNA required to induce *Xbra* expression at a given distance from the source of the signal is very similar whether it is supplied by the naturally inducing (vegetal) cells or by other (animal) cells which never normally release this signal.

It has recently been reported^{6,7} that the treatment of dissociated cells with activin protein fails to reveal a sharp concentration dependence of gene expression when this is assayed at an early time (after 2½ h), in the way that was previously described for assays at 15 h^{3,4}. Our results described above were obtained with conjugates cultured for 5–6 h and analysed at the same early stage (10½) as used by Green *et al.*⁶. When we tested *Xbra* expression at 1-h intervals from the time of conjugation, we saw no expression at 3½ h, but at 4½ h the light expression seen already revealed the same distance effect seen in Fig. 2a–c.

A minimum estimate of the rate at which this signalling process takes place comes from our observation that *Xbra* RNA can be seen 5 h after conjugation at 23 °C at a distance of at least 280 μm from the source. The rate of spread is therefore not less than 55 $\mu\text{m h}^{-1}$, and is almost certainly faster, because animal caps are not sufficiently large to determine the maximum distance that can be achieved, and because other genes are probably activated further from the source than *Xbra*.

Cell movement and cell division

Our results so far described establish the spread of an *Xbra*-inducing effect, but they do not establish its cellular basis.

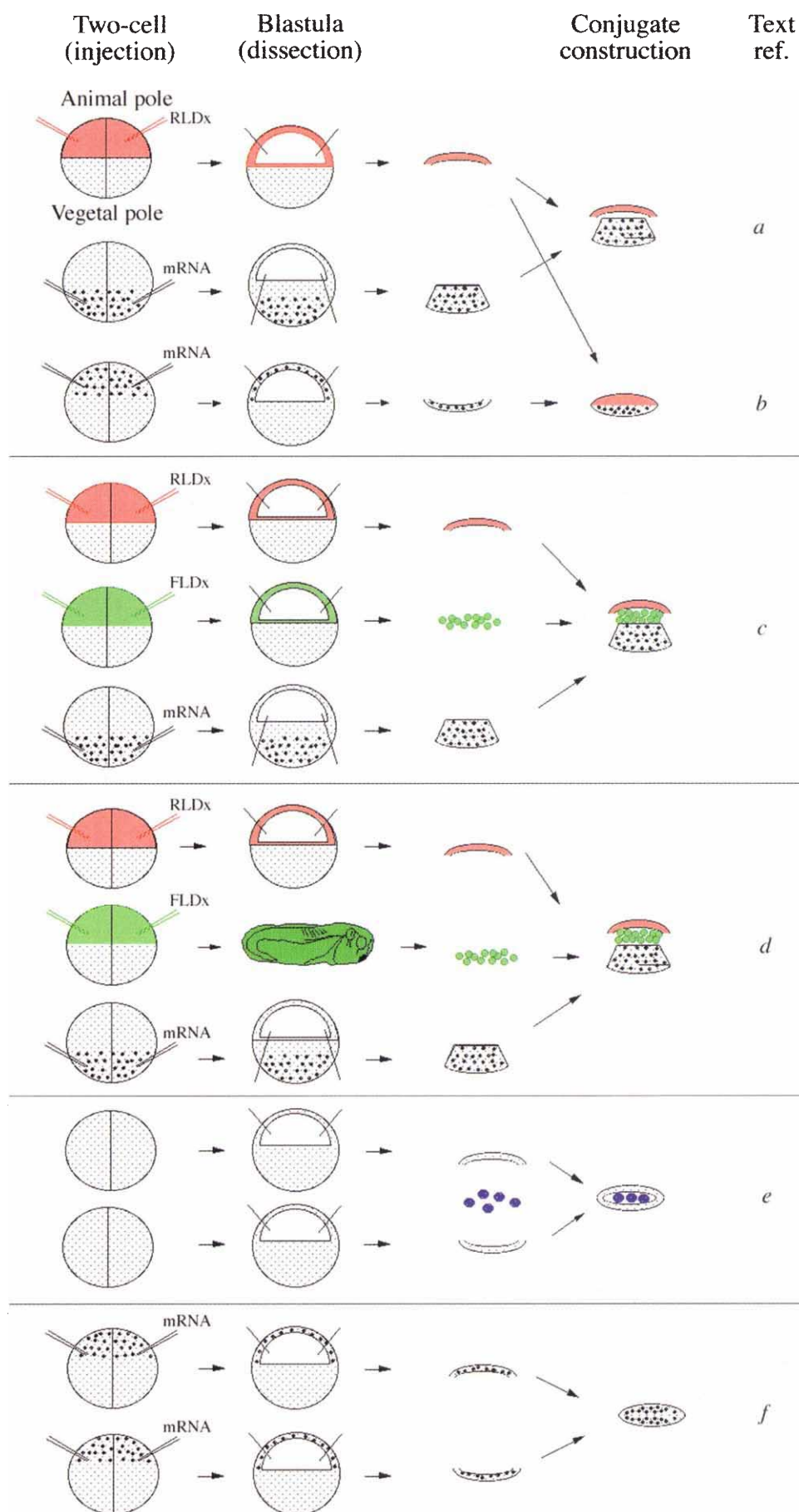


FIG. 1 *a–f*, Diagrams illustrating the design of experiments. See text for further details. Red, cells containing the lineage marker rhodamine lysinated dextran (RLDx); green, cells containing fluorescein lysinated dextran (FLDx). Black spots represent injected mRNA. Blue circles (in *e*) represent beads containing activin protein.

FIG. 2 Mesoderm and muscle gene expression in animal-vegetal conjugates of *Xenopus* blastulae. Conjugates were prepared at stage 8 by standard procedures¹⁷, and cultured until fixation at stage 11 (a-c, e) or stage 18 (d). After histological sectioning, slides were hybridized to a digoxigenin-labelled RNA probe specific for *Xbra*¹³ (a-c, e, purple), or stained with an anti-XMyoD antibody⁴⁷ (d, black nuclei). In a-c, the animal part of each conjugate was lineage-labelled with RLDx; *in situ* hybridization to sections reduces fluorescence to too low a level for photography, but fluorescence was present in all tissue above the dotted lines in a-c. The vegetal part of each conjugate was injected with activin mRNA: a, 2 pg; b, 7 pg; c, 20 pg. In a-c, the vertical lines indicate distances in μm of *Xbra* expression from mRNA-containing vegetal tissue. e, Section of a normal stage-11 embryo.

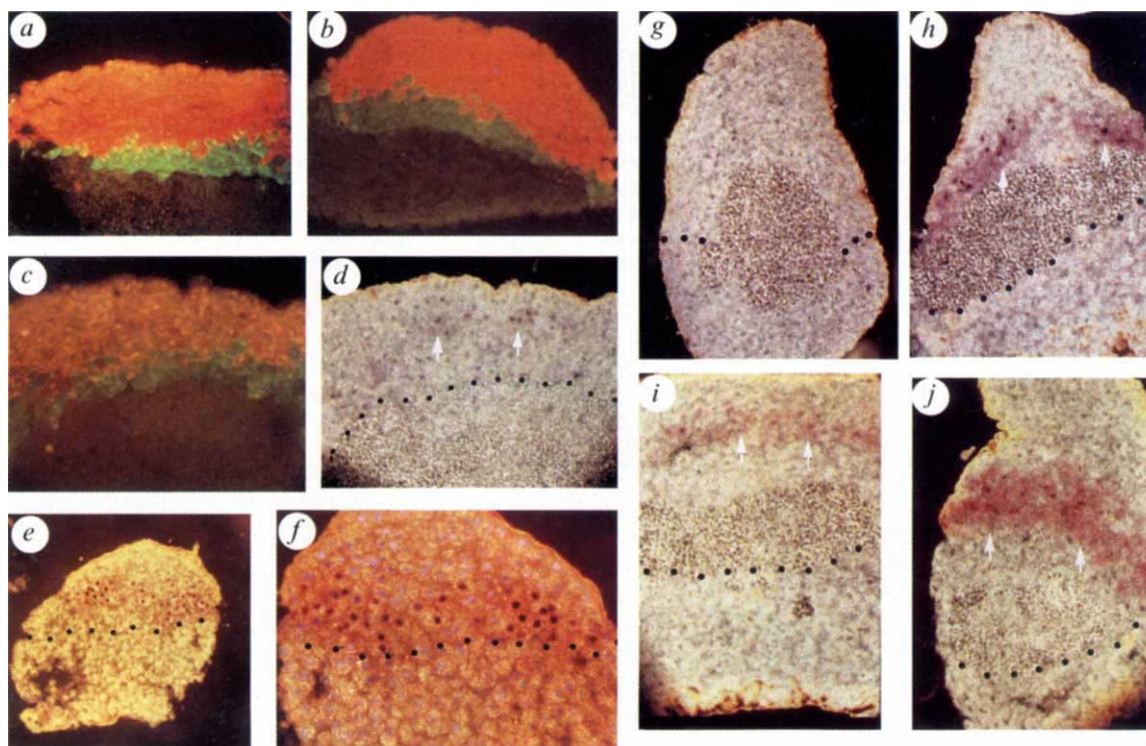
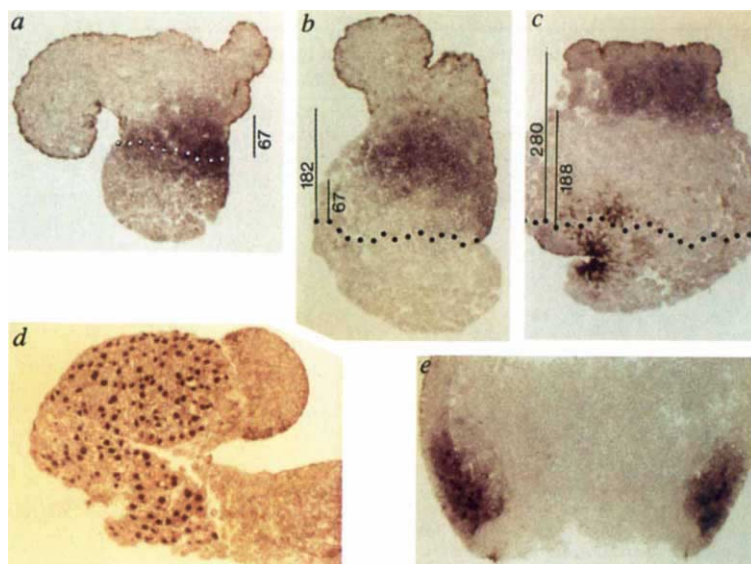


FIG. 3 *Xbra* expression in conjugates fixed at the early gastrula stage ($10\frac{1}{2}$). a-d, A layer of reaggregated FLDx-lineage labelled animal cap cells (green) was placed between vegetal tissue injected with 20 pg activin mRNA (brown) and FLDx animal caps (red). No significant cell movement has taken place (a, b), and *Xbra* staining, which is mainly nuclear at this stage, is seen in the red RLDx cell region (white arrows, d); c and d are the same sections photographed respectively before and after *in situ* hybridization. e, f, Conjugates composed of two animal caps, of which the one below the black dots contained mRNA but no lineage label; that above the dotted line contained RLDx but no mRNA. Conjugates were cultured in $5 \mu\text{g ml}^{-1}$ cytochalasin. *Xbra* nuclear stain-

ing is seen in a band (dark purple spots). g, h, Activin signalling induces *Xbra* expression after passing through non-competent cells. Stage-40 reaggregated endoderm cells, recognized by the refractile yolk platelets, do not induce *Xbra* in an animal cap sandwich (g), but permit signal transmission from activin mRNA-containing animal tissue (below the dotted line) to *Xbra*-expressing animal tissue not containing mRNA (above the refractile endoderm tissue, white arrows). i, j, Activin signalling can be transmitted by cycloheximide-inhibited stage-40 endoderm cells (same design as g, h). White arrows indicate *Xbra* expression (purple) in RLDx-labelled responding tissue.

One possibility is that the cells containing activin mRNA signal to the responding cells with which they are in direct contact, and these cells then move away to a distant position within the responding tissue. A variant of this idea is that a responding cell in contact with an inducing cell divides to generate daughters and granddaughters, which are now separated from the inducing cells. Both these explanations suppose

that the stronger the *Xbra* signal received, the further the induced cells or their daughters move away from the source of the signal.

The important principle behind these ideas is that the signalling process might be transmissible only by direct contact, and that the subsequent spatial relationship of induced cells would be established by cell movement and/or cell division. Alterna-

TABLE 1 The position of cells expressing *Xbra* is related to their distance from a source of activin

Inducing tissue	Activin mRNA (pg)	Xbra expression in animal cap cells: distance from activin mRNA source (μm)			MyoD expression
		Maximum*	Minimum†	Average‡	
Vegetal					
Mesoderm	20	280	110	214	–
Vegetal					
Mesoderm	7	170	33	102	+
Vegetal					
Mesoderm	2	140	0	62	++
Vegetal					
Mesoderm	NIL	80	0	35	–
Animal	20	260	124	187	
Animal	7	180	70	137	
Animal	2	30	0	10§	
Animal	NIL	0	0	0	

* Most distant point at which *Xbra*⁺ cells can be detected within the space of tissue available.

† Space in which no *Xbra* cells can be detected.

‡ Average distance also corresponds to the region of strongest *Xbra* expression.

§ Animal tissue containing activin mRNA was also *Xbra*⁺.

tively, the signalling process could involve the spread of a signalling molecule itself through cells that do not themselves move.

To distinguish these ideas, we have constructed conjugates with fluorescein (FLDx)-containing animal cap cells as a thin reaggregated layer between inducing vegetal cells and a rhodamine (RLDx)-containing animal cap (Fig. 1c). If cell movement is involved in generating the distance effect, we would expect the FLDx animal cap cells closest to a strong activin mRNA-vegetal cell source to move into the more distant RLDx-animal cell region. In fact, no such cell movement took place in conjugates fixed at the early gastrula stage (Fig. 3a, b), and *Xbra*-synthesiz-

ing cells are clearly located in the distal RLDx cells (Fig. 3c, d). Cell movement is not therefore responsible for the distance effect.

To determine the possible role of cell division, we have cultured conjugates in medium containing $5 \mu\text{g ml}^{-1}$ cytochalasin, a procedure previously found to inhibit cell division and cell movement⁸. Its effect is seen by the presence of multinucleated cells (not shown). Although cytochalasin reduces the distance over which signalling occurs, *Xbra* RNA is seen in cells separated by several cell diameters from the activin source (Fig. 3e, f).

We conclude that cell movement and cell division do not account for the separation of *Xbra*-expressing cells from the source of the inducer. Therefore this process must involve signal transmission through or past cells which do not themselves express *Xbra*. Cells that transmit, but do not seem to respond to, a signal can extend in our experiments for about 200 μm or six cell diameters from its source (Fig. 2c).

Signalling by passive diffusion

A fundamental question concerning intercellular signalling is whether transmission takes place by passive diffusion through or past cells, rather than by active amplification of the signal. In the experimental system under discussion here, those responding cells that are nearest to the activin mRNA-containing vegetal cells and which do not themselves express *Xbra* might nevertheless synthesize activin, and so pass the signal along to their neighbours by a relay process.

To distinguish passive diffusion from relay amplification we have inserted non-responding (that is, non-competent) cells between inducing vegetal and responding animal tissues (Fig. 1d). As non-competent cells, we have used endoderm (progenitor intestine) cells from a stage-40 tadpole, labelled with FLDx; these cells cannot induce *Xbra* in an animal cap sandwich

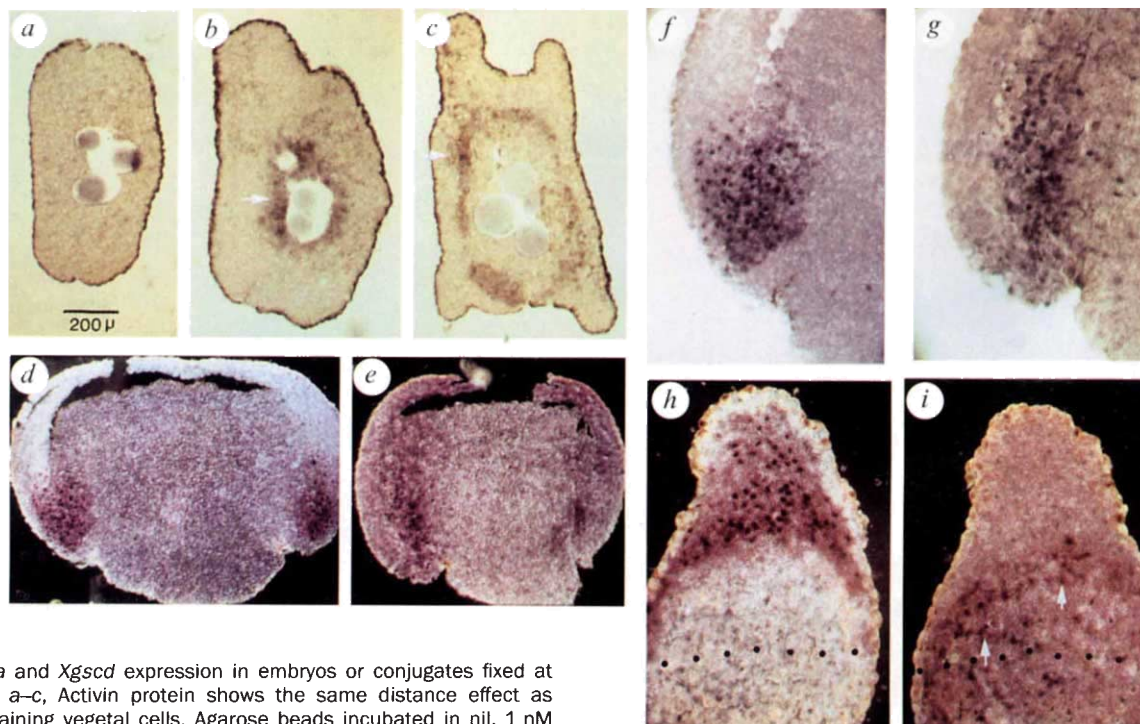


FIG. 4 *Xbra* and *Xgscd* expression in embryos or conjugates fixed at stage 10 $\frac{1}{2}$. a–c, Activin protein shows the same distance effect as mRNA-containing vegetal cells. Agarose beads incubated in nil, 1 nM or 4 nM activin protein were included in animal cap sandwiches and processed for expression of *Xbra* RNA (white arrows). In subsequent experiments, smaller amounts of a more active preparation of activin gave similar results. d–g, Comparison of *Xbra* (d, f) and *Xgscd* (e, g) expression in normal stage 10 $\frac{1}{2}$ embryos, analysed by *in situ* hybridization of digoxigenin probes to sections. h, i, Adjacent sections of the same animal-animal conjugate stained for *Xbra* (h) or *Xgscd* (i). The mRNA-injected animal tissue extends downwards from the dotted lines,

the upper, responding animal cap (above the dotted lines) having been RLDx-lineage labelled. In h, the strong nuclear and diffuse purple cytoplasmic stain for *Xbra* is seen to be above and well separated from the signalling cells below the dotted line. In i, the region above the dotted line, unstained in h, contains *Xgscd*-stained nuclei (white arrows). There is a partial overlap of *Xbra* and *Xgscd* staining cells.

(Fig. 3g), nor can they express *Xbra* in response to mesoderm-inducing signals. Nevertheless the *Xbra*-inducing signal from activin mRNA vegetal cells is transmitted successfully through the non-competent cells (Fig. 3h).

This leaves open the possibility that the non-competent cells might respond to their adjacent activin mRNA-containing vegetal cells by synthesizing some other (non-activin) inducer, which causes *Xbra* expression in distant cells but not in themselves. This possibility has been tested by inhibiting protein synthesis in the layer of non-competent cells. Previous work has shown that a 1-h treatment with cycloheximide at $5 \mu\text{g ml}^{-1}$ inhibits protein synthesis for a period of 4 h, after which it slowly recovers^{9,11}. Conjugates were therefore constructed with a layer of stage-40 endoderm cells which had been incubated in $10 \mu\text{g ml}^{-1}$ cycloheximide for 2 h, and these were placed in a layer between inducing and responding tissues (Fig. 1d). *Xbra* was expressed in the responding animal cells (RLDx-labelled), but not by the cycloheximide-inhibited endoderm cells which are readily recognized by their content of yolk platelets (Fig. 3i, j).

We conclude that the long-range signalling capable of activating *Xbra* can be transmitted passively through non-responding cells, or more likely past these cells via the extracellular matrix. It has not been possible to distinguish passive diffusion from relay amplification in other experimental systems.

Activin protein can mediate spatial control

Although distant *Xbra* expression in our experiments is dependent on overexpressing activin mRNA in the inducing cells, it is not clear whether activin protein is directly responsible for the distance effect, or whether this might be achieved through some other component released by the message-injected cells. To resolve this uncertainty, we have inserted agarose beads loaded with activin protein into animal cap sandwiches (Fig. 1e). At a low activin dose, we see *Xbra* gene expression in a ring of cells close to the activin-containing beads (Fig. 4a, b). When beads are loaded with activin at a much higher concentration, *Xbra* expression is moved to 100–200 μm or up to six cell diameters away from the source (Fig. 4c). Therefore we can reproduce, with purified activin protein, the same relationship between the concentration of inducer and the location of gene activation as we have described for cells injected with activin mRNA. This supports our view that the biological activity of these mRNA-containing cells results from the release and spread of activin protein itself. We should however emphasize that signalling induced by mRNA-injected cells rather than by protein-loaded beads approximates more closely to the normal mesoderm induction process.

Activation of *Xgscd* compared with *Xbra*

We next investigated whether genes other than *Xbra* can be induced by the signalling process we have described. In particular, we wish to know which, if any, genes are expressed in the considerable space between a strong activin mRNA source and the nearest *Xbra* RNA, as seen in Fig. 2c. *Xgoosecoid* (*Xgscd*) is one of several genes expressed in only the dorsal lip region of an early gastrula¹². Using *in situ* hybridization to sectioned material¹³, a procedure we prefer to whole-mount hybridization on account of the more even access to deep tissues, we see *Xgscd* expression, like that of *Xbra*, as a diffuse cytoplasmic and strong nuclear stain (Fig. 4e). In normal early gastrulae, *Xbra* staining is precisely localized to an equatorial ring (Fig. 4d, f), whereas *Xgscd* is expressed in a different but partly overlapping region on the dorsal but not ventral side (Fig. 4e, g, dorsal side on the left).

Conjugates which include animal or vegetal tissue with large amounts (20 pg or more) of activin mRNA were sectioned alternately onto two slides, which were reacted with *Xbra* or *Xgscd* antisense RNA probes. As expected, *Xbra* was expressed at a distance from the signalling tissue; however, *Xgscd* was expressed in the intervening but partially overlapping space

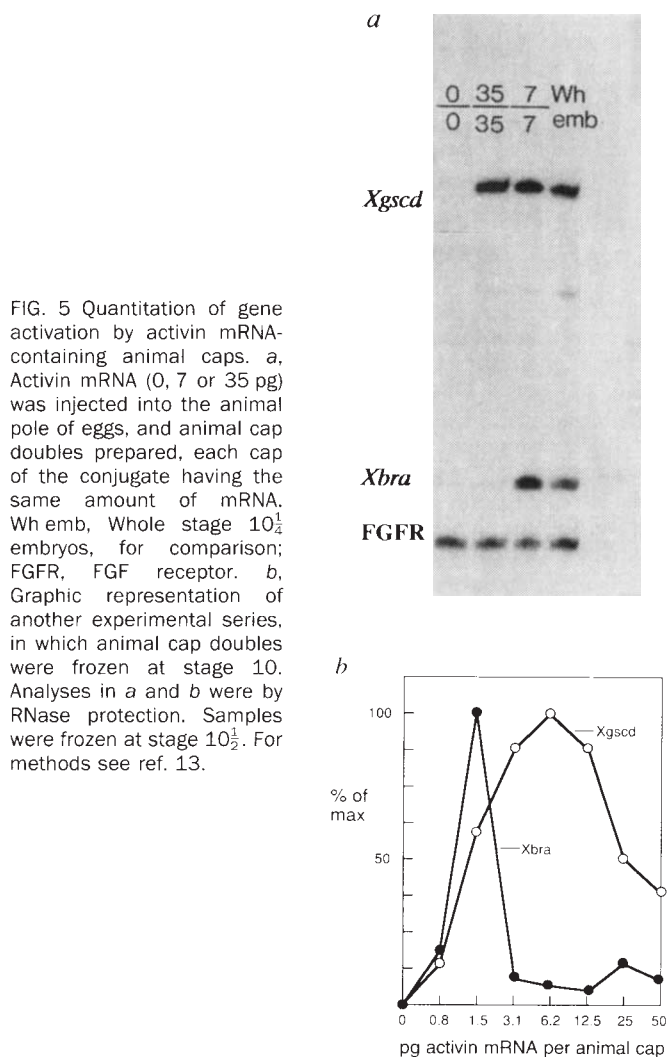


FIG. 5 Quantitation of gene activation by activin mRNA-containing animal caps. **a**, Activin mRNA (0, 7 or 35 pg) was injected into the animal pole of eggs, and animal cap doubles prepared, each cap of the conjugate having the same amount of mRNA. Wh emb, Whole stage 10½ embryos, for comparison; FGFR, FGF receptor. **b**, Graphic representation of another experimental series, in which animal cap doubles were frozen at stage 10. Analyses in **a** and **b** were by RNase protection. Samples were frozen at stage 10½. For methods see ref. 13.

between the activin source and cells expressing *Xbra* (Fig. 4h, i), a pattern expected in view of the normal expression of these genes. This result supports the proposition that activin, or other morphogens, activates the most dorsal responding genes nearest its source where its concentration is presumed to be highest.

To determine the relative expression of *Xbra* and *Xgscd* in a more quantitative way than can be achieved by *in situ* hybridization, we have used RNase protection. To simplify interpretation, we have analysed conjugates in which both component tissues were prepared in the same way, for example by injection of high, low or nil levels of activin mRNA (Fig. 1f'). Thus we might predict that *Xgscd* would be expressed most strongly in animal caps with the greatest amount of activin mRNA, whereas *Xbra* should be most strongly represented in middle-dose activin mRNA samples. This is in fact the result obtained (Fig. 5a), because relative to normal embryos, the expression of *Xgscd* is stronger after activin than after no activin mRNA injection, whereas *Xbra* is 15 times stronger after a middle (7 ng) than a high (35 ng) mRNA injection. In two more extensive experiments (not shown), activin mRNA was injected in small increments from 0 to 50 pg per animal cap, and conjugates were analysed after 5 and 7 h of incubation. *Xgscd* expression was greatest after injection of 3–12 pg activin mRNA, whereas at both time points *Xbra* was expressed most strongly at 1.5 pg of activin mRNA, and was virtually eliminated by higher doses (Fig. 5b).

In summary, our *in situ* results show that the expression of *Xbra* and *Xgscd* is spatially related to the source of an inducing

signal. In all, we have discussed three levels of response to activin signalling. These are *Xgscd* nearest the signal source, *Xbra* in the middle distance, and furthest away a nil response probably corresponding to the normal ectodermal fate of uninduced ectoderm. Our experiments therefore provide direct support for the hypothesis that gene activation is determined by the spatial relationship of cells to a source of morphogen.

Activin signalling in normal development

Does activin signalling play an important part in *Xenopus* development? Cells of the second tier of a 32-cell *Xenopus* embryo are major contributors to the mesoderm and its derived cell types^{14,15}, but require contact with third-tier cells to become mesoderm¹⁶. For this and many other reasons^{17–20}, it is believed that second-tier cells and their daughters are normally induced by vegetal cells to be redirected from an epidermal to a mesodermal fate, by the so-called Nieuwkoop induction. Several gene products are able to mediate this process, and activin is one of the strongest candidates for a natural inducer^{21–23}. Although its mRNA is not synthesized until well after the Nieuwkoop induction has started²⁴, an activin-like protein is present in eggs²⁵ (its distribution not known). Activin A was the first mesoderm inducer to be purified from normal cell extracts²⁶, and has mesoderm-inducing activity at a very low concentration (0.2 ng ml⁻¹ of purified XTC-MIF²⁴, or about 10 pM activin). Even if activin is not a natural inducer, it is probable that a closely related molecule such as Vg1 may be^{27,28}. It is therefore very likely that the effects and mechanism of activin signalling described here are an important part of the normal mesoderm-forming processes in early development.

Intercellular signalling in other systems

We draw three conclusions from our results. The first is that signals emanating from cells can initiate new gene activity and a new pathway of cell differentiation in other cells located at least 10 cell diameters away, and this takes place in solid tissue of a normal embryo. In the vertebrates, this conclusion has been implied by many embryonic induction experiments since the time of Spemann and Mangold's dorsal lip grafts²⁹, as well as by the ectopic expression of *Wnt* genes^{30,31}. In *Drosophila*, *wingless* (*wg*) has effects on cells, and its proteins can spread, a few cell diameters from its source^{32,33}.

A second more important result of our experiments is to strongly support the operation of a morphogen gradient, an idea suggested by other work. In *Drosophila*, it has been shown that a change in the concentration of *bicoid* protein determines the position of nuclei expressing *hunchback*^{34,35}. In this case, how-

ever, signalling takes place in a syncytium by the spread of RNA or protein through cytoplasm. In contrast, *decapentaplegic* (*dpp*) encodes an intercellular signalling molecule belonging, like activin, to the TGF- β family of growth factors, and is thought to be distributed in a dorso-ventral gradient during the post-syncytial stage of development³⁶. When *dpp* RNA is injected into syncytial embryos, 2–4-fold increases in concentration lead to the formation of progressively more dorsal structures³⁷. It has been suggested that *hedgehog* (*hh*) may also act as a morphogen in the patterning of the *Drosophila* epidermis³⁸, though its mode of action may be indirect³⁹. Within the vertebrates, evidence has accumulated in favour of the idea that the zone of polarizing activity in the chick limb bud is a source of morphogen whose concentration determines the type of digit to be formed⁴⁰. The implantation of preloaded beads into a limb bud generates a gradient of retinoic acid that is related to the sequence of digits formed⁴¹, though not in any simple way to the early expression of genes⁴². In amphibia, increasing amounts of *Xgscd* can dorsalize ventral tissue in a dose-dependent way⁴³; however, as a transcription factor, *Xgscd* (like *bicoid*) is a response to, and not an agent of, intercellular signalling, and might be a downstream consequence of the activin effects we report here. Our own results on activin concentration agree with those of Green and Smith^{3,4} and extend them most significantly by showing that the concentration of activin released by signalling cells determines which cells in a responding field of normal solid tissue will express a particular type of gene. Our results therefore provide direct evidence for the importance of a morphogen gradient in normal development.

The third conclusion from our work concerns the mechanism by which signalling molecules exert their effect through a mass of cells. In previous work it has not been possible to determine whether the long-range effects of *wg* or *hh* result from diffusion of a signalling molecule or from a cascade or relay mechanism⁴⁴. In an entirely different system, Placzek *et al.*⁴⁵ have shown that a chemoattractant emanating from the floor plate of a neural tube can spread through neural epithelium over a distance of 250 μ m and can cause a change in the orientation of growing axons towards the source of the influence. Our results reported here with non-competent cells in which protein synthesis was inhibited seem to provide the strongest evidence so far that a peptide growth factor can progress past many cells and activate genes by a diffusion rather than a relay or cascade mechanism, generating a concentration gradient as it does so.

Overall, our results add direct support to the operation of a morphogenetic gradient in vertebrate development, an idea which has been prominent in the minds of embryologists for many years⁴⁶. □

Received 2 June; accepted 15 August 1994.

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ACKNOWLEDGEMENTS. We thank the Cancer Research Campaign for supporting this work. P.H. was supported by the Fulbright Commission, and P.L. by ICI/Churchill College and EEC Human Mobility Fellowships. We thank A. Martinez-Arias, D. St Johnston and P. A. Lawrence for comments on the manuscript, and E. Tweed and R. Coulson for technical assistance.

An upper limit on the density of low-mass stars in the Galactic halo

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DESPITE the evidence for substantial amounts of dark matter in the haloes of galaxies^{1,2}, its nature is still unknown. Gravitational microlensing of light from a nearby galaxy by objects in the Galactic halo with masses of about 0.1 solar masses was reported recently^{3,4}. If these objects are baryonic, low-mass stars seem the most probable candidates. But extrapolation of the locally observed distribution of stellar masses⁵⁻⁷ to this range suggests that such low-mass stars comprise an insignificant fraction of the Galactic halo, so that microlensing events due to 0.1-solar-mass stars should be rare. Here we report the results of a search for very low-mass stars at high galactic latitudes. Using their near-infrared colours to estimate their intrinsic luminosities, we conclude that very little of the dark matter in the halo of the Milky Way can be made of low-mass hydrogen-burning stars. If the dark matter is baryonic, then we predict that further searches for microlensing events will see a large number of events corresponding to masses of less than 0.07 solar masses.

Existing optical CCD (charge-coupled device) studies of the low-mass stellar content of the Galactic halo suggest that low-mass luminous stars are not a major contributor to the mass of the luminous stellar halo, to mass limits of 0.14 solar masses ($0.14M_{\odot}$; ref. 8). Limits based on optical plate searches⁶ constrain the stellar density at an absolute bolometric magnitude of $M_{\text{bol}} = 13.5$ mag ($\sim 0.08\text{--}0.10M_{\odot}$) to $\sim 2 \times 10^{-3} \text{ pc}^{-3} \text{ mag}^{-1}$. To this magnitude there is no sign of the upturn in the luminosity function which would be required to produce a significant dynamical mass. In searching for stars at or below $0.1M_{\odot}$ it is best to use the near-infrared, because their very red colours limit optical searches to small distances. Here we describe a search in the K' (2.1 μm), I (8,400 $\text{\AA}$) and B (4,400 $\text{\AA}$) bands for very red objects over a significant area (103 arcmin²) of sky to a moderately deep limiting magnitude ($K' = 19$ mag), which allows us to extend this result to $M_{\text{bol}} = 15.5$ mag ($\sim 0.07\text{--}0.09M_{\odot}$).

The data are drawn from four elongated strips of sky obtained in B , I and K' in the course of the galaxy survey reviewed in ref. 9, and which is more extensively discussed by Songaila *et al.*¹⁰. The observations were made at the University of Hawaii 2.2-m telescope in K' , with the NICMOS-3 256×256 pixel camera¹¹ (with 2:1 reducing optics, giving 0.75-arcsec pixels), whereas the I - and B -band observations were made with a Tektronix 2,048 CCD with a pixel size of 0.22 arcsec. The strips are at high galactic and ecliptic latitudes but widely separated in right ascension (RA). For studies of faint stars in the local neighbourhood, these high-galactic-latitude fields avoid contamination by more distant disk stars, and thus are well-suited for the present work. A typical strip is shown in Fig. 1. Each strip consists of one 10 arcmin (RA) $\times$ 3 arcmin (declination, dec.) area of sky. K' and I magnitudes were measured in 4-arcsec diameter apertures throughout the central regions of the strip, giving 103 arcmin² of total sky coverage. In all cases the 5σ limiting K' depth exceeds $K' = 20$ mag (where σ is the noise of the background night sky over the aperture; typically $5\sigma = 20.5$ mag in the K' band), but in order to ensure a very robust sample with well-determined colours we chose to limit the current work to $K' \leq 19$ mag. At these magnitudes $\sim 10\%$ of the objects are stars, and $\sim 90\%$ galaxies for the observed galactic latitudes. The plot of $(I - K')$ against K' for the 519 galaxies and stars brighter than $K' = 19$ mag is shown in Fig. 2 with $\pm 1\sigma$ error bars in the $(I - K')$ colour. All objects to the $K' \leq 19$ mag limit are detected above the 1σ I limit of 24.5 mag, but we do note that it is the I -band depth which makes the major contribution to the colour uncertainty.

Before considering the limits on redder ($I - K > 5$) stars where there are no well studied objects for comparison, it is worth considering the $4 < (I - K) < 5$ range, where we can use a more direct interpretation rather than relying on extrapolations or model comparisons. From Fig. 1 we can see that there are eighteen objects in the $K' < 19$ mag sample with $4 < (I - K) < 5$, but only three of these are morphologically compact, with the rest being galaxies. Some of these objects may also be unresolved galaxies. From Leggett's compilation¹², $(I - K) = 4.5$ corresponds to stars with absolute K magnitude $M_K \approx 10$ for both young disk and old disk stars, and these would be visible to ~ 600 pc. For a uniform halo component this then places an upper limit on the space density of $4 < (I - K) < 5$ stars of $5 \times 10^{-3} \text{ pc}^{-3}$, or a density of less than $6 \times 10^{-3} \text{ pc}^{-3}$ per unit bolometric magnitude at $M_{\text{bol}} = 13.5$ mag using the $(I - K)$ versus M_{bol} relations of ref. 6. The upper limit from stars in this colour

FIG. 1 One of the four fields used in the analysis. (Central coordinates; RA 3 h 54 min 58.5 s, dec. $+01^{\circ} 01' 01''$ (1950).) The bottom panel shows the K' image of the field and the corresponding Kron-Cousins I -band image is given in the upper panel. The K' magnitudes were calibrated using Elias standards²¹ and the I magnitudes with faint Landolt standards²². Comparison with 12-arcsec diameter aperture magnitudes on isolated stars gives an average correction of ~ 0.1 mag from 4-arcsec diameter aperture magnitudes to total magnitudes in both bands. The images have been boxcar-smoothed with a 1.5-arcsec smoothing and are contoured at levels rising by factors of 2 from a surface brightness of 21.2 mag arcsec⁻² in K' and 24.5 mag arcsec⁻² in I . The lowest levels are at the 14σ and 9σ levels respectively for the smoothed images, so that all visible objects are real on both frames.

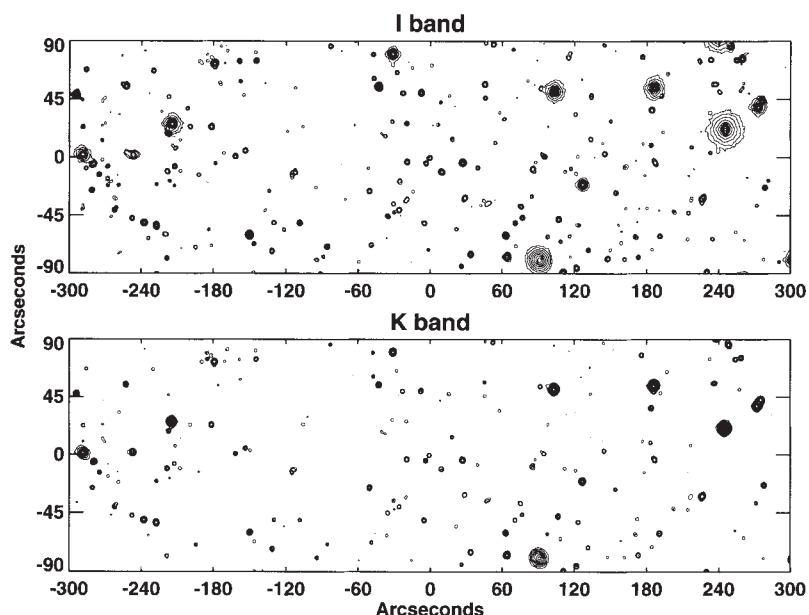
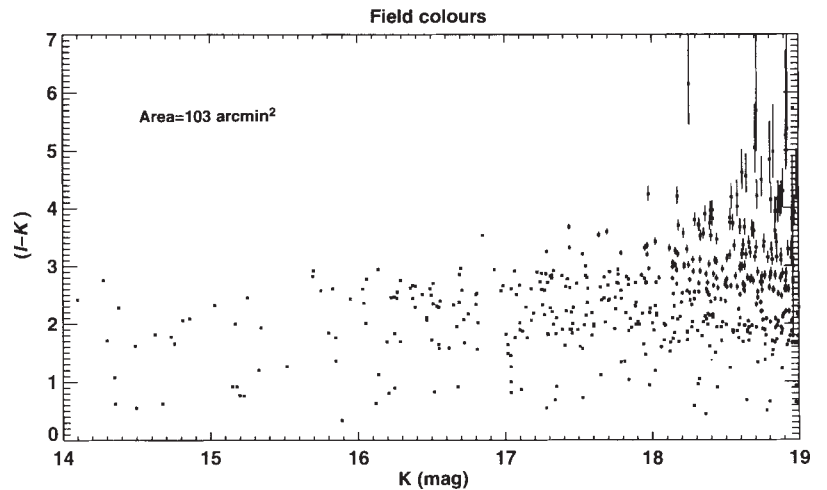


FIG. 2 Plot of $(I-K')$ against K' for the $K' \leq 19$ mag sample. The sample of 519 objects includes both stars and galaxies. In high-galactic-latitude fields, such as the ones chosen here to avoid contamination by more distant disk stars, $\sim 10\%$ of the objects are stars and $\sim 90\%$ are galaxies. 1σ error bars in $(I-K')$ are plotted for all points.



range is weaker, but consistent with Tinney, Mould and Reid's optically determined values⁶ of $2 \times 10^{-3} \text{ pc}^{-3}$ per unit bolometric magnitude at these magnitudes.

The reddest compact objects in the fields, based on $(I-K')$ colour, are potentially lower-mass stars. Tinney, Mould and Reid⁶ have shown an empirical monotonic relation between colour and magnitude, M_K , finding that an $(I-K')$ colour of 5 corresponds roughly to $M_K = 10.8$ mag ($M_{\text{bol}} = 14$ mag). Linearly extrapolating M_K against $(I-K')$, an $(I-K')$ colour of 6 corresponds to $M_K = 11.6$ mag ($M_{\text{bol}} = 15$ mag). With this extrapolation the present sample magnitude limit of $K' = 19$ corresponds to a limiting distance of 440 pc for $(I-K') = 5$ and 300 pc for $(I-K') = 6$.

There are very few extremely red objects in the fields; only five objects have $(I-K') > 5$. Three of these are extended and two are pointlike. Only one spatially extended object has $(I-K') > 6$. Only three objects with colours this red are known, from work other than the present survey. All of these appear, on the basis of follow-up imaging with detailed spectral study, to be galaxies¹³. The reddest known star is GD165B discovered by Becklin and Zuckerman¹⁴ with $(I-K) = 5.08$ (ref. 15). If we adopt the two compact objects detected at $(I-K') = 5-6$ as upper limits on the stellar population (again these may, in fact, be unresolved galaxies), we obtain a stellar number-density per unit magnitude interval $n(M_{\text{bol}} = 14.5) \leq 8 \times 10^{-3} \text{ pc}^{-3} \text{ mag}^{-1}$. At $(I-K') = 6-7$ we detect no candidate objects and also place a limit of $n(M_{\text{bol}} = 15.5) \leq 1.2 \times 10^{-2} \text{ pc}^{-3} \text{ mag}^{-1}$. Thus we conclude that for the halo population of interest the luminosity function drop which is seen between $M_{\text{bol}} = 10$ and $M_{\text{bol}} = 13.8$ (ref. 6) continues to $M_{\text{bol}} = 15.5$ mag. An upturn to values as high as $3 \times 10^{-2} \text{ pc}^{-3} \text{ mag}^{-1}$ (roughly comparable to the peak of the luminosity function at $M_{\text{bol}} = 10-11$) can be excluded. $M_{\text{bol}} = 15.5$ mag corresponds roughly to $0.07 M_{\odot}$ stars^{16,17}, for near solar metallicity.

The above assumptions are appropriate for stars with near-solar metallicities. Leggett¹² has summarized the optical and infrared colours of cool stars with metallicities appropriate to young disk ($[\text{Fe}/\text{H}] \approx 0$), old disk, and halo ($[\text{Fe}/\text{H}] \sim -1$ to -2) populations. Figure 13 in ref. 12 shows the translation from $(I-K)$ to M_1 that is critical for this Letter. The shift in colours for moderately red stars can be compared with our Fig. 3, which shows a colour-colour plot with our data compared with a disk metallicity main sequence. The points offset from the main sequence toward red $(I-K')$ are galaxies, while those along the main sequence are stars; in selected subregions of these fields, nearly all the objects have been spectroscopically observed, confirming this separation. The star-galaxy separation in the $(B-I)$ versus $(I-K')$ colour-colour plane is a potentially important advantage in studying faint populations although we do not draw on it here.

For very faint and red stars, Leggett's Fig. 13 (ref. 12) implies that the metallicity effects tend to damp out, but also makes clear how little evidence there is to bear on this issue. For the purposes of this Letter, we have adopted the point of view that the metallicity effects do not significantly influence our arguments, but we acknowledge that future studies of low-metal, very-low-mass stars may show this viewpoint to be incorrect.

A still more extreme possibility is that the lensing objects belong to extreme population III, with metallicities far lower than are observed for typical low-mass halo stars. Figure 3 shows the colour-colour track for zero metallicity dwarfs, from Saumon *et al.*¹⁸. A $0.1 M_{\odot}$ star lies at the blue $(B-I)$, red $(I-K')$ end of this track; our data show there are no population objects less massive than $0.1 M_{\odot}$. Because $M_1 = 11.1$ mag at $0.092 M_{\odot}$.

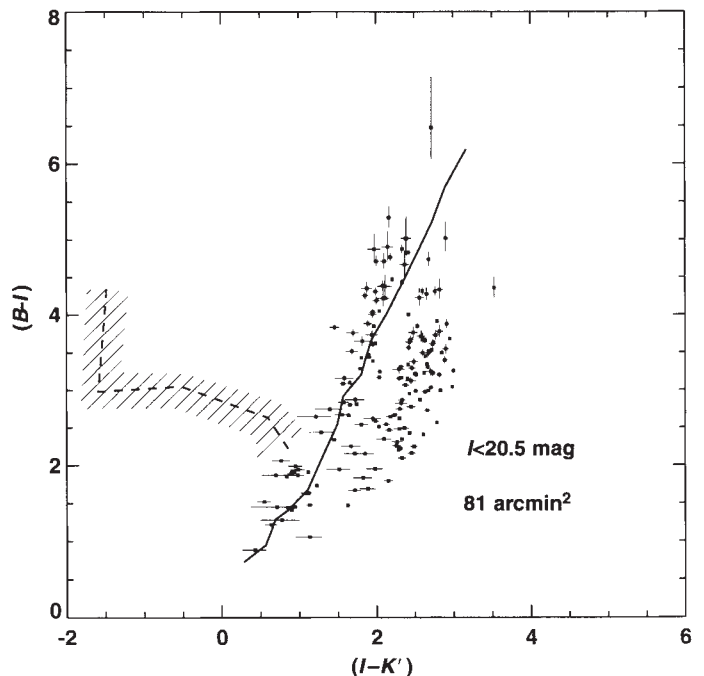


FIG. 3 Colour-colour plot of $(B-I)$ against $(I-K')$ for an $I \leq 20.5$ mag selected sample covering 81 arcmin². The colours of the objects are plotted with $\pm 1\sigma$ error bars in both colours, and stars are seen to lie along the main-sequence colours²³⁻²⁵, shown with the heavy solid line (and with an average colour correction to K' mag (ref. 26)). Galaxies lie to the right of the star tracks in these plots. The cross-hatched region surrounding the dashed curve shows the locus of colours for zero-metallicity stars¹⁸ with temperatures between 2,000 and 3,800 K, which falls at bluer $(I-K')$ colours than the field population, in an unoccupied region of the colour-colour plane.

for these objects¹⁸, our data place an upper limit of $1.2 \times 10^{-3} \text{ pc}^{-3}$ for zero-metallicity stars of this mass in a uniform halo, that is, a limit more stringent than the upper limits derived above for stars of disk or halo metallicity.

Because our conclusions are based on four spatially separate fields, they should prove an unbiased sampling of the halo, free from biases due to clumping or other structure. Our number-density limits eliminate any possibility that stars at or near the hydrogen-burning limit contribute any significant fraction of the dynamical mass of the halo, irrespective of metallicity.

Turning again to the $(I-K') > 6$ data, we have compared the absence of such objects with the expectation based on the K-band luminosity function derived by Nelson *et al.*¹⁹ under the assumption of constant star formation rates but with a variety of mass functions (IMFs). Adopting their $T_{\text{max}} = 1,500 \text{ K}$ curves, where T_{max} is the maximum effective temperature of stars contributing to the luminosity function, as very roughly corresponding to an $(I-K') > 6$ colour selection we find that, scaling their standard model to the field galactic latitudes (typically around 45°), the expected number of $(I-K') > 6$ stars integrated to $K' = 19 \text{ mag}$ is $1.2 \times (\rho / (0.10 M_\odot \text{ pc}^{-3}))$, where ρ is the local density associated with dark matter, and is relatively insensitive to the functional form of the IMF or the choice of T_{max} . The current sample therefore places a 1σ upper limit of $0.08 M_\odot \text{ pc}^{-3}$ on the local brown-dwarf mass density under the Nelson *et al.*¹⁹ assumptions. Further searches of larger areas are clearly needed to refine this result, and are currently being carried out by one of us (J.-S.H.).

We conclude that little of the halo dark mass can be in hydrogen-burning stars with masses $\geq 0.1 M_\odot$ even if these objects are of low metallicity¹⁷. It appears unlikely that any stellar remnant population can be present in sufficient numbers to account for a significant fraction of the local mass density without violating other astrophysical constraints, and in any case no mechanism to create stellar remnants of such low mass is known. Thus, unless the possible MACHO events (of refs 3 and 4) are attributable to some exotic (and possibly hitherto unknown) objects which are not composed of baryonic matter in its normal state as hydrogen and helium, and so do not become luminous stars, identification of these occurrences, if indeed due to gravitational lensing, is most likely to be with brown dwarfs below the hydrogen-burning limit of $0.07 M_\odot$. This mass limit is the approximate limit of current observational extrapolations, as well as a natural fiducial break in physical processes describing the stellar mass function. This explanation has a clear prediction: if the MACHO events are real and caused by compact substellar masses of ordinary baryonic matter, then we predict that as the experiments progress they will see many events of shorter average duration than was seen in the initial detection^{3,4}. Very recently, Sahu²⁰ suggested that the MACHO events may have resulted from lensing by stars in the Large Magellanic Cloud, rather than objects in the halo of our Galaxy. This is consistent with our own conclusions, in that we see no evidence for halo stars of the mass estimated for the MACHO lenses. $\square$

Received 3 November 1993; accepted 24 August 1994.

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A loop-top hard X-ray source in a compact solar flare as evidence for magnetic reconnection

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SOLAR flares are thought to be the result of magnetic reconnection—the merging of antiparallel magnetic fields and the consequent release of magnetic energy. Flares are classified into two types¹: compact and two-ribbon. The two-ribbon flares, which appear as slowly-developing, long-lived large loops, are understood theoretically^{2–6} as arising from an eruption of a solar prominence that pulls magnetic field lines upward into the corona. As the field lines form an inverted Y-shaped structure and relax, the reconnection of the field lines takes place. This view has been supported by recent observations^{7–10}. A different mechanism seemed to be required, however, to produce the short-lived, impulsive compact flares. Here we report observations made with the Yohkoh¹¹ Hard X-ray Telescope¹² and Soft X-ray Telescope¹³, which show a compact flare with a geometry similar to that of a two-ribbon flare. We identify the reconnection region as the site of particle acceleration, suggesting that the basic physics of the reconnection process (which remains uncertain) may be common to both types of flare.

The flare studied here occurred close to the west limb of the Sun in the active region NOAA 6994 on 13 January 1992 17:25 UT. It was a GOES M2.0-class flare in soft X-rays and showed a single-spike time history in hard X-rays at $\geq 20 \text{ keV}$. The spike had a duration of $\sim 1 \text{ min}$ full-width at half-maximum (FWHM), peaking at 17:28:10 UT. The Hard X-ray Telescope (HXT) and Soft X-ray Telescope (SXT) images are shown in Fig. 1, from which we identify the following essential components. (1) A soft X-ray flaring loop seen in soft X-rays at $\sim 2 \text{ keV}$. We see three bright patches, one at the loop apex and the other two at the ends (footpoints), although the northern footpoint is relatively weak. The intensity variation along the loop, however, is relatively small. The intensity variations along as well as across the loop are mainly due to variations in emission measure ($n_e^2 V$) where n_e is the number density of electrons and V is the volume, not to variations in electron temperature (T_e). Here, the T_e and $n_e^2 V$ distributions are derived from intensity comparisons between images taken through two different passbands in the 1–2-keV range. The HXT low-energy (14–23 keV) source matches the soft X-ray loop. (2) A high-temperature region above the soft X-ray loop. Electron temperatures are derived to be $\sim 2 \times 10^7 \text{ K}$. The intensity is weak because of the small emission measure. (3) Double footpoint sources seen in hard X-rays. They

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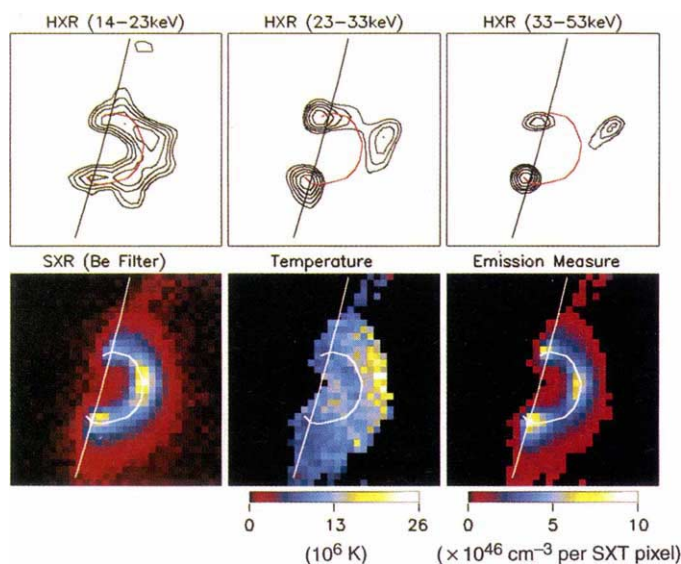


FIG. 1 Hard X-ray (top row) and soft X-ray (bottom row) images of the 13 January 1992 flare that occurred near the west solar limb. HXT images in three HXT energy bands (namely L, M1-, and M2-bands; 14–23–33–53 keV) are shown at the top for the photon count accumulation interval 17:26:52–17:27:39 UT (rising phase). Contour levels are 70.7, 50.0, 35.4, 25.0 and 17.7% of the peak intensity for each map. At the bottom from left to right are an SXT 119- μ m Be-filter image (intensity map) taken at 17:27:01 UT, an electron temperature map, and an emission measure map. The latter two are derived from intensity comparison between the Be-filter image and a 11.6- μ m Al-filter image taken at 17:27:07 UT. The field of view of each frame covers 78.4×78.4 arcsec or 5.7×10^4 km by 5.7×10^4 km. The SXT pixel size is 2.45×2.45 arcsec ($\sim 1,800 \times 1,800$ km). Solar north is to the top, west to the right. For positional reference, the solar limb and the backbone of the soft X-ray flaring loop are shown by solid lines. Accuracy of coalignment is estimated to be within ~ 1 arcsec.

are dominant at ≥ 25 keV and located at the footpoints of the soft X-ray loop. The hard X-rays are bremsstrahlung from energetic electrons accelerated near the loop apex and streaming down along the loop toward both ends^{14,16}. These sources are also seen in soft X-rays^{17,18}. (4) A 'loop-top' hard X-ray source located well above the apex of the soft X-ray loop. Its centroid position is significantly higher than the latter by more than ~ 10 arcsec or $\sim 7,000$ km. (The angular resolutions of SXT and HXT are ~ 3 and ~ 5 arcsec respectively.) This source occupies only a small portion of the high-temperature region and has no direct counterpart in the SXT images.

The loop-top hard X-ray source varies rapidly in timescales less than a few tens of seconds and similarly to the footpoint sources (Fig. 2). In addition, judging from the hard X-ray intensities in the different energy bands, the source has a relatively hard spectrum, only slightly softer than those of the footpoint sources. This source only appears for a short interval. Later we see a different class of hard X-ray source near the soft X-ray loop apex which is characterized by a more gradual time variation and a softer spectrum. We call the early hard source a 'loop-top impulsive source' for clarity. Whether this source is of thermal or nonthermal origin is not clear because of lack of detailed hard X-ray spectral information. Because we can perceive a slight spectral softening towards higher energies, it may be interpreted as thermal bremsstrahlung. If this is the case, we get $T_e \sim 2 \times 10^8$ K and $n_e n_i V (\sim n_e^2 V) \sim 5 \times 10^{43}$ cm $^{-3}$, where n_i is the ion density of the ambient plasma.

The existence of an impulsive hard X-ray source above the soft X-ray flaring loop is of crucial importance. First, it informs us that something energetic is going on outside the bright soft X-ray loop. Second, this something is directly related to particle acceleration. According to Sakao¹⁶, typical double footpoint

sources change their intensity simultaneously within a few tenths of a second, which suggests that electrons responsible for the footpoint X-ray emission are accelerated near the loop apex. Taking this into account, it is most likely that we are observing the particle acceleration site directly in the loop-top impulsive source. Finally, the soft and hard X-ray images combined together strongly suggest a magnetic-field geometry with a current sheet in which magnetic reconnection is in progress during the time interval of primary energy release.

The hypothetical scenario depicted in Fig. 3 differs only in detail from standard pictures¹⁹ for two-ribbon flares. For convenience we assume the loop-top impulsive hard X-ray source to be thermal in origin. This corresponds to a maximum value for n_e ; if n_i is much larger than n_e the derived value of n_e must be reduced proportionally. Based on the thermal assumption, we get a density $\sim 3 \times 10^8$ cm $^{-3}$, a total number of electrons (or ions) $\sim 3 \times 10^{35}$, and a thermal energy content $\sim 3 \times 10^{28}$ erg for the hard X-ray emitting 2×10^8 -K plasma. (Similarly SXT observations give a density $\sim 2 \times 10^{10}$ cm $^{-3}$ for the 2×10^7 -K plasma at the same location. It is not clear, however, whether or not the two components, one seen with HXT and the other with SXT, occupy the same volume.) We speculate that the 2×10^8 -K plasma is created as a result of shock heating. This would be possible if the reconnection outflow velocity exceeds $\sim 3,000$ km s $^{-1}$. This velocity is not surprisingly large; an Alfvén velocity of 3,000 km s $^{-1}$ requires a magnetic field intensity of 200 G even when the density is 2×10^{10} cm $^{-3}$. This intensity is not unreasonable in an active region corona.

The injection rate of electrons (>20 keV) into the footpoint sources, evaluated by adopting the thick target model^{20,21}, is $\sim 2 \times 10^{35}$ s $^{-1}$ at the peak time (an energy deposition rate of $\sim 1 \times 10^{28}$ erg s $^{-1}$), using the measured photon power-law index of ~ 4 . This injection rate suggests a high efficiency of acceleration; if we suppose that the electrons stream down from the loop-top source, it means that almost all electrons in the 2×10^8 -K plasma would escape in a second or so. Or more probably, the nonthermal electrons are produced directly from the shock in parallel with the creation of the 2×10^8 -K plasma. In either case, the reconnection outflow impinging on the loop must have a higher density than that estimated for the 2×10^8 -K plasma; from continuity, a density greater than $\sim 2 \times 10^9$ cm $^{-3}$ is required for a shock area $\sim 1 \times 10^{18}$ cm 2 and a shock velocity 3,000 km s $^{-1}$.

In this scenario, the high-temperature region seen in soft X-rays may be on the periphery of the reconnection outflow, where the outflow velocity is smaller so that only a weak shock

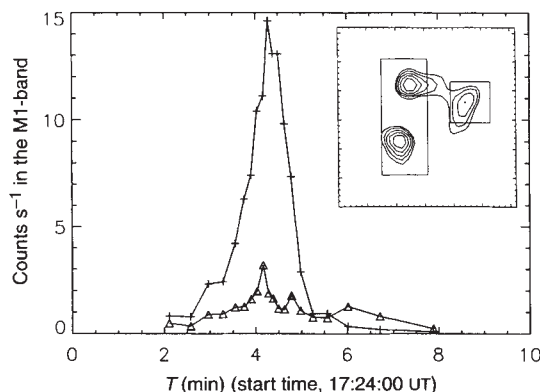


FIG. 2 Temporal behaviour of the loop-top (crosses) and double footpoint (triangles) sources in the HXT M1-band. The integrated intensities are estimated for the two boxed areas, that is, one for the loop-top source and the other for the double footpoint sources (inset). It is clear that the loop-top source varies almost coincidentally with the double footpoint sources. The integrated intensities are given in units of HXT photon counts averaged over its 64 detectors (subcollimators) whose average geometrical aperture is ~ 1 cm 2 .

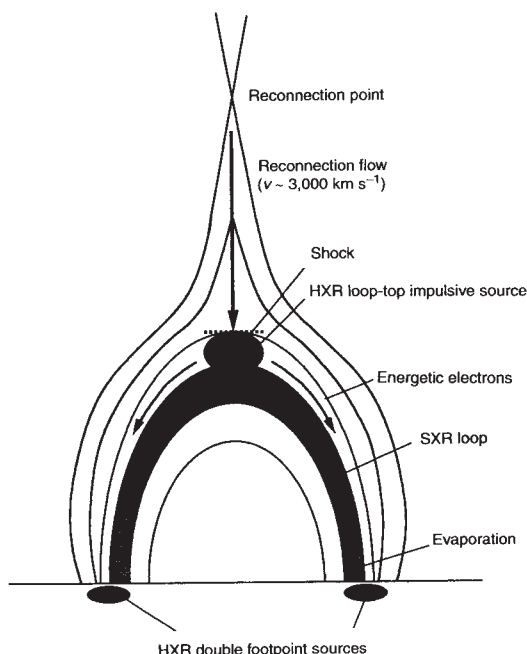


FIG. 3 The magnetic-field geometry for reconnection derived from the present observation. The important features are: elongated antiparallel magnetic fields above an arcade of closed loops; a current sheet (or neutral sheet) formation between them and reconnection of antiparallel magnetic fields. An outflow or jet from the reconnection point impinges on the underlying closed loop and forms a shock, resulting in a high-temperature ($T_e \sim 2 \times 10^8$ K) region just above the closed loop. It is also likely that electrons are accelerated in the shock and stream down along the reconnected field towards the double footpoint sources.

is formed and no strong energization occurs. The soft X-ray flaring loop may represent previously reconnected magnetic fields filled with material evaporated from the dense chromosphere. Perhaps the evaporation is caused not only by energetic electron precipitation but also by heat conduction. In any case, it is a byproduct of the primary energy release.

The observations reported here have revealed for the first time the existence of a loop-top impulsive hard X-ray source in a compact solar flare. There are other examples of this type of source in the Yohkoh data base²². We believe that these observations may simplify our theoretical view of solar-flare energy release. The data strongly suggest that we have identified the site of the powerful acceleration of energetic electrons common to solar flares, namely a coronal current sheet. It may now be possible for magnetic reconnection on macroscopic scales to explain compact flares as well as two-ribbon flares. Our discussion of these observations invokes shock formation resulting from the reconnection jet expected in a current-sheet configuration. This discussion was more illustrative than quantitative; it will be instructive to pursue a more thorough analysis of both Yohkoh and ground-based data on this and related flares. □

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ACKNOWLEDGEMENTS. We thank H. Hudson for help in polishing the manuscript. The Yohkoh satellite is a Japanese national project, launched and operated by ISAS involving many domestic institutions, with multilateral international collaboration with US and UK.

A photorefractive polymer with high optical gain and diffraction efficiency near 100%

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PHOTOREFRACTIVE materials are of considerable interest for the development of all-optical devices¹. The photorefractive effect appears in materials that exhibit an electric-field-dependent refractive index and that are photosensitive, such that the spatial distribution of photogenerated charge carriers is modified on irradiation with light. The diffraction pattern formed by the interference of two coherent light beams within such a material generates a non-uniform internal electric field that in turn modulates the refractive index. The resulting refractive-index pattern forms a grating that can diffract light and thereby give rise to two-beam coupling, whereby one of the writing beams gains energy at the expense of the other—a property that can be exploited in photonic devices. Although the best photorefractive materials currently available are inorganic crystals such as LiNbO₃, there is considerable interest in the development of photorefractive polymers^{2–8}, owing to their structural flexibility, ease of processing and lower cost. We describe here a polymer composite with excellent photorefractive properties. We have achieved a diffraction efficiency approaching 100% and a net two-beam coupling gain of more than 200 cm⁻¹, making these polymeric materials suitable for immediate application in areas such as dynamic holographic storage and optical information processing¹.

In organic materials, the properties required for the photorefractive effect, including photosensitivity, photoconductivity and electro-optic response, are provided by different molecules. As a result, the properties can be optimized separately, unlike in inorganic photorefractive crystals such as LiNbO₃. Our investigations were performed on a polymer composite (see Fig. 1) based on the photoconductor poly(*N*-vinylcarbazole) (PVK). Composites using PVK were the first polymers to show efficient photorefractivity⁶ and are still among the organic materials with the best performances^{4,7}. Photosensitivity in the visible was provided by adding a small amount of 2,4,7-trinitro-9-fluorenone (TNF), which forms a charge-transfer complex with PVK. Crucial for the steady-state performance of a photorefractive composite is the electro-optic chromophore. The latter should possess a large dipole moment μ , a large first hyperpolarizability β , and at the same time a small absorption coefficient α at the operating wavelength. The electro-optic chromophore should be highly soluble in the composite to obtain a strong photorefractive effect. Furthermore, as will be shown below, a large aniso-

Received 12 May; accepted 19 August 1994.

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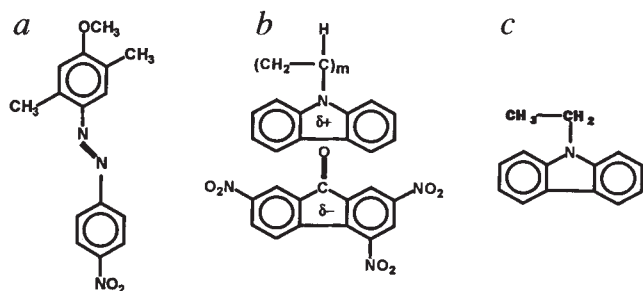


FIG. 1 Chemical structures of the compounds used in the photorefractive composite: a, 2,5-dimethyl-4-(*p*-nitrophenylazo)anisole (DMNPAA); b, charge-transfer complex of poly(*N*-vinylcarbazole) (PVK) with 2,4,7-trinitro-9-fluorenone (TNF); c, *N*-ethylcarbazole (ECZ).

tropy $\Delta\alpha^*$ of the linear polarizabilities parallel and perpendicular to the molecular axis of this chromophore is beneficial. We chose 2,5-dimethyl-4-(*p*-nitrophenylazo)anisole (DMNPAA), which satisfies these requirements.

To obtain a macroscopic electro-optic effect in the material, the originally randomly oriented electro-optic chromophores have to be aligned by an electric field ('poling'). Besides the alignment of the chromophores, the field also assists the charge photogeneration by reducing the recombination probability and provides the net force for the drift of the carriers. In order to be able to pole the material at room temperature, *N*-ethylcarbazole (ECZ) was added to decrease the glass transition temperature T_g of the material. The composition was DMNPAA:PVK:ECZ:TNF 50:33:16:1 wt%; the concentration of the nonlinear chromophore was significantly higher than in other reported photorefractive polymers (typically ≤ 33 wt%)²⁻⁸. Uniform films of ~ 105 μm thickness were fabricated by sandwiching the composite between two transparent electrodes, the field thus being applied perpendicular to the sample surface.

Because of the charge separation in photorefractive materials, a phase shift occurs between the original light fringe pattern and the generated index grating. This phase shift is a unique feature of the photorefractive effect, distinguishing it from any other mechanism that gives rise to a light-induced refractive-index modulation. It enables energy transfer between two coherent light beams, the so-called 'two-beam coupling'.

We performed two-beam-coupling experiments to verify the non-local nature of the index gratings, and degenerate four-wave-mixing (DFWM) experiments to test the total grating amplitude. The experimental geometry was identical for both techniques and is shown in Fig. 2 inset. Two coherent 'writing beams' were overlapped in the sample to create a fringe pattern. They were both either 's' or 'p'-polarized (perpendicular or parallel, respectively, to the plane defined by the incoming beam and the sample normal). In order to have a non-zero component of the external field along the grating wave vector $\mathbf{K}$, the experiments were performed in a tilted geometry. The grating period was 3.1 μm . In the DFWM experiments, index gratings recorded in the photorefractive material were probed by a weak beam, counterpropagating with the writing beam 1 (Fig. 2 inset) and the intensity of the transmitted and the diffracted light were monitored. In the two-beam-coupling experiments, the counter-propagating beam was absent and the transmission of the two writing beams was measured. All experiments were performed using a single continuous-wave laser diode (9 mW output, wavelengths $\lambda = 675$ nm).

The two-beam-coupling results depend on both the index grating amplitude and its phase shift. As shown in Fig. 2, the steady-state gain coefficient for p-polarized beams Γ_p increases monotonically with the external field E , yielding $\Gamma_p = 220$ cm^{-1} at $E = 90$ $\text{V } \mu\text{m}^{-1}$. The gain by far exceeds the absorption in the sample at this voltage ($\alpha = 13$ cm^{-1}), giving a net optical gain of $\Gamma_{p,\text{net}} = 207$ cm^{-1} . For s-polarized beams, on the other hand, the beam,

which gained energy when it was p-polarized, now loses energy for the identical field direction with $\Gamma_s = -40$ cm^{-1} at 90 $\text{V } \mu\text{m}^{-1}$ (Fig. 2). This is due to opposite signs of the index modulations sensed by the two polarizations.

The DFWM experiments were performed with s-polarized writing beams in order to keep the beam coupling small and to reduce the effects of two-beam energy coupling between the writing beams which causes the grating phase and amplitude to vary throughout the sample⁹. Unlike the gain coefficient Γ , the diffraction efficiency η depends solely on the amplitude of the index grating, not on its phase. Figure 3 shows the DFWM results for p-polarized readout. η_p increases with the electric field and reaches a maximum of 86% at $E = 61$ $\text{V } \mu\text{m}^{-1}$, and the light is completely diffracted. Further increase of the field leads to periodic energy transfer between the diffracted and the transmitted beam; at $E = 81$ $\text{V } \mu\text{m}^{-1}$, all light is again directed into the original probe wave. The sum of diffracted and transmitted signals gradually decreases as the external field increases ($\Delta\alpha = 5$ cm^{-1} at 90 $\text{V } \mu\text{m}^{-1}$). This is due to electric-field-induced absorption changes in the sample¹⁰ as we verified by an independent transmission measurement in the absence of the writing beams. Similar experiments were performed for s-polarized readout (not shown). The diffraction efficiency is smaller than for p-polarized readout, and no maximum was observed for fields up to 90 $\text{V } \mu\text{m}^{-1}$.

The dynamics of grating formation are complex. The speed depends on the applied electric field, the light intensity and the

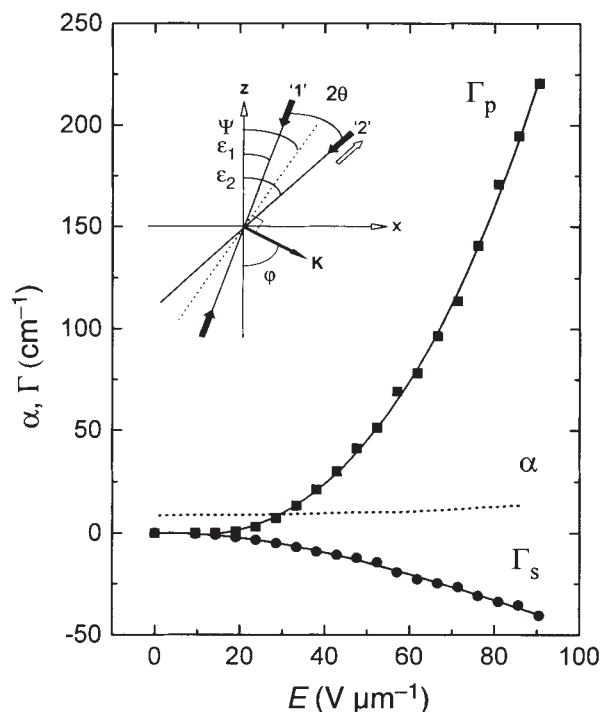


FIG. 2 Two-beam-coupling experiment in DMNPAA:PVK:ECZ:TNF (power density of the pump beam, 1 W cm^{-2}). Main Figure, external-field dependence of the gain coefficient Γ for s- (beam ratio $b = 1.5$; circles) and p-polarized writing beams ($b = 13$; squares). b is the original intensity ratio of the writing beams, defined as $b = (I_2(I_1 = 0))/(I_1(I_2 = 0))$; Γ is calculated from the amplification factor $\gamma = I_1(I_2 \neq 0)/I_1(I_2 = 0)$ measured experimentally using $\Gamma = \{\cos \varepsilon_1 \ln [b\gamma/(b+1-\gamma)]\}/d$, where $I_{1,2}$ are the beam intensities of the writing beams, the indices '1' and '2' denote the amplified and the diminished beam, respectively, and ε_1 is the angle of the amplified beam with respect to the sample normal. Also shown is the absorption coefficient α (dashed line). The solid lines are guides to the eye. Inset, write/readout geometry. The angle between the writing beams is $2\theta_{\text{ext}} = 22^\circ$ and the tilt angle is $\psi_{\text{ext}} = 60^\circ$. Inside the material, $2\theta = 7^\circ$ and $\psi = 29^\circ$; the bulk refractive index was measured (by ellipsometry) as $n = 1.75$.

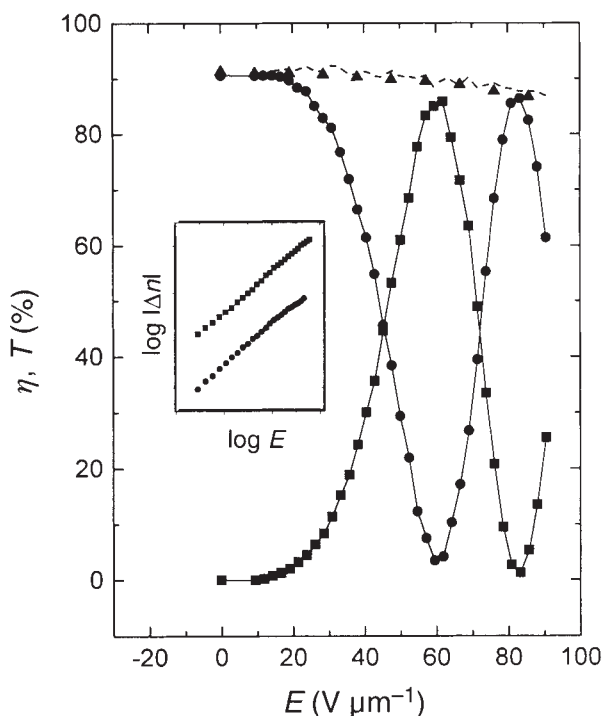


FIG. 3 Degenerate four-wave-mixing experiment in DMNPAA:PVK:ECZ:TNF with s-polarized writing beams (power density, 1 W cm^{-2} ; beam intensity ratio $b=1.3$) and a p-polarized reading beam (power density, 0.35 mW cm^{-2}). Main Figure, external-field dependence of the diffraction efficiency η defined as the intensity ratio of the diffracted probe light and the probe before entering the sample (squares) and the transmission in the presence (circles) and in the absence (triangles), respectively, of the writing beams. The dashed line is the sum of diffracted and transmitted intensity in the presence of the writing beams, indicating that the maximum achievable diffraction efficiency is limited by absorption and reflection losses ($\sim 12\%$). The solid lines are guides to the eye. Inset, log-log plot of the external-field dependence of the index grating amplitudes $|\Delta n_s|$ (circles) and $|\Delta n_p|$ (squares) for s- and p-polarized readout, respectively, obtained from the coupled-waves model for slanted lossy dielectric transmission gratings¹¹, taking into account the change in absorption with field, but not the small phase and amplitude variations caused by energy exchange between the writing beams.

grating spacing. Under our experimental conditions (see legends of Figs 2 and 3) and at $90 \text{ V } \mu\text{m}^{-1}$ the diffraction efficiency rises to $\sim 95\%$ of the maximum value within $\sim 100 \text{ ms}$, reaching a steady-state value after 10 s . The efficiency of the grating drops to 15% of the maximum value within 24 h after all beams and the electric field were switched off. The recorded index pattern can be erased by uniform illumination.

From the DFWM data we calculated the absolute total index grating amplitude in the material $|\Delta n_{s,p}|$ for s- and p-polarized readout, respectively, using Kogelnik's coupled-waves model¹¹ (see Fig. 3 legend). The observed periodic energy transfer between the transmitted and the diffracted beams in the DFWM experiments is in accordance with this model. The log-log plot of $|\Delta n_{s,p}|$ versus the electric field is shown in Fig. 3 inset. At $E=81 \text{ V } \mu\text{m}^{-1}$ the index grating amplitudes are calculated to be $|\Delta n_s|=1.5 \times 10^{-3}$ and $|\Delta n_p|=5.5 \times 10^{-3}$, respectively. The anisotropy ratio $|\Delta n_p/\Delta n_s|=3.7$ is nearly independent of the applied field. Our two-beam-coupling experiments reveal that Δn_s and Δn_p have opposite signs (Fig. 2), that is, $\Delta n_p/\Delta n_s=-3.7$.

To verify that such large field-induced refractive-index changes can be achieved in this material, we carried out ellipsometric measurements in the absence of the writing beams and with the probe polarized at 45° with respect to the vertical axis.

Approximately at the fields where the extrema appear in the DFWM experiment we observed that the phase delay between the s- and p-components of the incident light was π and 2π , respectively. The phase delay is given by $\phi=[2\pi d(\Delta n_p-\Delta n_s)]/\lambda \cos \varepsilon_1$ (where d is the sample thickness and ε_1 is defined in Fig. 2), leading to $\Delta n_p-\Delta n_s=5.7 \times 10^{-3}$ at $E=81 \text{ V } \mu\text{m}^{-1}$. This is smaller than the value derived from the DFWM experiments for the same field strength ($\Delta n_p-\Delta n_s=7.1 \times 10^{-3}$) because of the smaller tilt angle ($\varepsilon_{1,\text{ext}}=50^\circ$) for the ellipsometry measurements. It is noted that these measurements verify that the space-charge field is smaller than the component of the external field along the grating wave vector ($E_{\text{SC}} \leq E_{\text{dc}} \sin \varepsilon_1$).

The η and Γ values found in our composite material are (by far), the highest reported for organic photorefractive materials to date. They originate from large refractive-index modulations in the material. To find the origin of these excellent properties we further analysed the anisotropy ratio $\Delta n_p/\Delta n_s$. It depends upon the microscopic properties of DMNPAA ($\Delta \alpha^*$, β , μ) and the properties of the host matrix such as its dielectric constant, that is, local field corrections have to be taken into account¹¹. For the electro-optic (EO) effect the anisotropy ratio for light polarizations parallel and perpendicular to the local poling-field direction is always positive¹³, $(\Delta n_{\parallel}^{\text{EO}}/\Delta n_{\perp}^{\text{EO}}) > 0$. The negative sign of $\Delta n_p/\Delta n_s$ thus indicates that effects other than the electro-optic effect play an important role in our material.

Because of the low T_g of the material, the electro-optic chromophores are mobile in the polymer matrix. The samples are poled during the recording by the total electric field E_{tot} , which is the superposition of the uniform external field E_{dc} and the non-uniform internal space-charge field E_{SC} ($E_{\text{tot}}=E_{\text{dc}}+E_{\text{SC}}$), producing a spatially varying orientation of the molecular dipoles in the material. Thus, not only the electro-optic coefficient, but also the linear birefringence of the material is modulated, yielding a stronger photorefractive effect than in materials with a fixed structure such as permanently poled polymers. This phenomenon was discussed recently by Moerner *et al.*¹⁴ as the 'orientational enhancement' mechanism. The anisotropy ratio for the electric-field-induced birefringence (BR) is always negative¹³, $(\Delta n_{\parallel}^{\text{BR}}/\Delta n_{\perp}^{\text{BR}}) < 0$. Assuming that the total index change is the sum of the individual changes by the birefringence and the electro-optic effect, the negative sign of $\Delta n_p/\Delta n_s$ unambiguously shows that the contribution of birefringence to the total index modulation is significant.

Our results offer a significant advance in the new field of research on photorefractive polymers. The excellent performance of the composite DMNPAA:PVK:ECZ:TNF can be explained by the high chromophore content, and by its low T_g , allowing the periodic orientation of the chromophores by the non-uniform internal field. This leads to refractive index gratings due to the birefringence and the electro-optic effect, and therefore to an enhanced photorefractive effect. The results provide evidence that the birefringence is mostly responsible for the excellent photorefractive properties of our material. Contributions from higher-order phenomena cannot be completely excluded. Diffraction from absorption gratings can be safely neglected because the maximum field-induced absorption change of 5 cm^{-1} gives an estimated diffraction efficiency of $\eta \approx (\Delta \alpha d/4)^2 \approx 1.5 \times 10^{-4}$. The improved quality of the films allows the application of high electric fields, enabling the observation of complete diffraction and large optical gain using a low-power laser diode. □

Received 25 April; accepted 24 August 1994.

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ACKNOWLEDGEMENTS. This work was supported by the USAF Office of Scientific Research and by the US National Science Foundation. K.M. thanks the Deutsche Forschungsgemeinschaft for a postdoctoral fellowship.

Transient nature of CO₂ fertilization in Arctic tundra

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THERE has been much debate about the effect of increased atmospheric CO₂ concentrations on plant net primary production^{1,3} and on net ecosystem CO₂ flux^{3–10}. Apparently conflicting experimental findings could be the result of differences in genetic potential^{11–15} and resource availability^{16–20}, different experimental conditions^{21–24} and the fact that many studies have focused on individual components of the system^{2,21,25–27} rather than the whole ecosystem. Here we present results of an *in situ* experiment on the response of an intact native ecosystem to elevated CO₂. An undisturbed patch of tussock tundra at Toolik Lake, Alaska, was enclosed in greenhouses in which the CO₂ level, moisture and temperature could be controlled²⁸, and was subjected to ambient (340 p.p.m.) and elevated (680 p.p.m.) levels of CO₂ and temperature (+4 °C). Air humidity, precipitation and soil water table were maintained at ambient control levels. For a doubled CO₂ level alone, complete homeostasis of the CO₂ flux was re-established within three years, whereas the regions exposed to a combination of higher temperatures and doubled CO₂ showed persistent fertilization effect on net ecosystem carbon sequestration over this time. This difference may be due to enhanced sink activity from the direct effects of higher temperatures on growth^{16,29–33} and to indirect effects from enhanced nutrient supply caused by increased mineralization^{10,11,19,27,34}. These results indicate that the responses of native ecosystems to elevated CO₂ may not always be positive, and are unlikely to be straightforward. Clearly, CO₂ fertilization effects must always be considered in the context of genetic limitation, resource availability and other such factors.

Net ecosystem carbon balance is the difference between gross primary productivity and losses from plant and soil respiration (together with generally small losses to herbivores). In the historical and recent geological past (Holocene epoch), net pri-

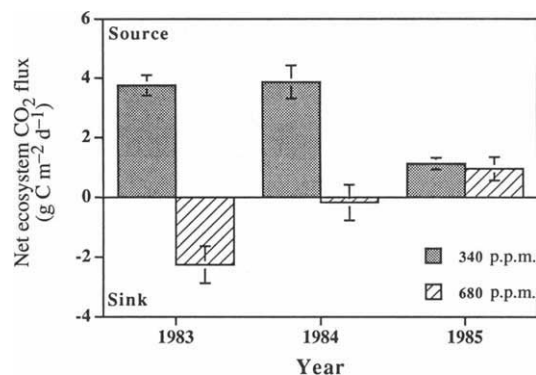


FIG. 1 Seasonal CO₂ flux of tussock tundra (dominated by *Eriophorum vaginatum*) ecosystems exposed to 340 and 680 p.p.m. CO₂ during the 1983–85 growing seasons at Toolik Lake, Alaska. Data are means $\pm$ 1 standard error; $n=3$ chambers per treatment.

mary productivity of permafrost-dominated northern ecosystems often exceeded heterotrophic respiration due to the cold, wet soil environment^{34,35}. As a result, these ecosystems during the Holocene were net sinks for carbon with respect to the atmosphere of up to ~ 0.1 to 0.3 petagrams of carbon per year (Pg C yr^{-1})^{34,40}.

Recent measurements of CO₂ flux in Arctic ecosystems indicate that tussock and wet sedge tundra are now sources of CO₂ to the atmosphere^{34,41,42} possibly due to changes in site water balance^{34,40,41,43} following the recently reported increase in northern-latitude surface temperatures^{44,48}.

Given the huge below-ground carbon stocks in Arctic soils, the release of CO₂ from Arctic ecosystems may exert a positive feedback on atmospheric CO₂ levels and greenhouse warming⁴¹. Conversely, under conditions of elevated CO₂ and global climate change, northern ecosystems could become a sink for carbon, providing a negative feedback on atmospheric CO₂ levels³⁴. The ultimate role of Arctic ecosystems in the global carbon budget largely depends on both the short- and long-term ecosystem response to elevated CO₂ and concomitant climate change.

Higher atmospheric CO₂ has the potential to increase plant growth in a variety of ways, including greater photosynthesis, higher water-use efficiency, depression of respiration, delayed leaf senescence, and relief of nutrient stress via enhanced nutrient-use efficiency, nitrogen fixation and nutrient uptake^{3,8,9,16,49}. There is, however, great uncertainty about whether these responses will hold for periods of years in natural

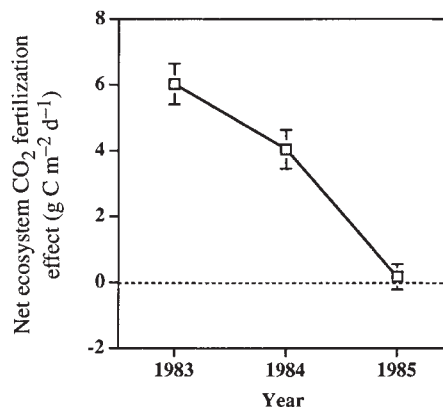


FIG. 2 The CO₂ fertilization effect over three growing seasons at Toolik Lake. Indicated is the absolute stimulation of net ecosystem CO₂ flux by a doubling of atmospheric CO₂ concentration (from 340 to 680 p.p.m.). The CO₂ fertilization effect is calculated as the difference between the flux measured at double and ambient CO₂ levels. Data are means $\pm$ 1 standard error; $n=3$ chambers per treatment.

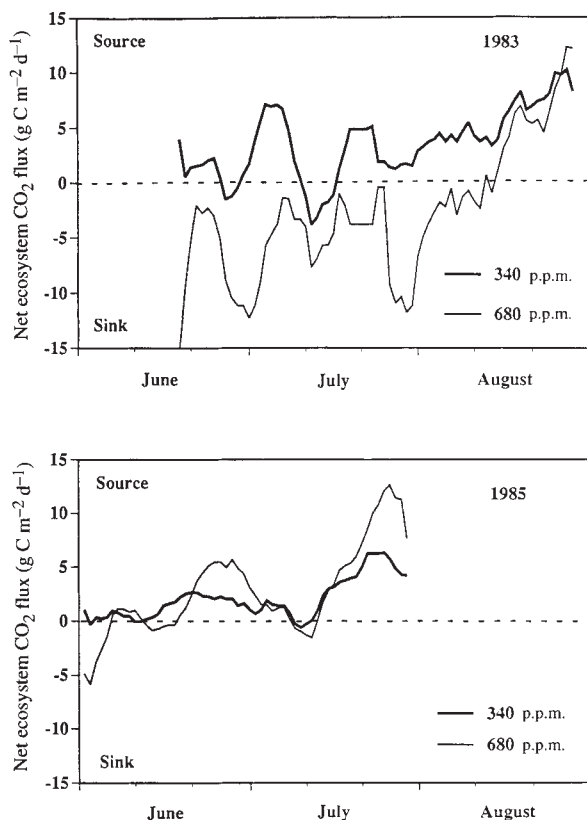


FIG. 3 Daily net ecosystem CO₂ for *in situ* tussock tundra at 340 and 680 p.p.m. CO₂, for 1983 (top) and 1985 (bottom). Data presented are 5-day sliding-mean values calculated from 6-minute average CO₂ fluxes throughout the period indicated. A computer malfunction caused atypically low temperatures for the season, and termination of this phase of the experiment in late July 1985.

ecosystems, and it has been suggested that limitations of water, nutrients and light will prevent most unmanaged ecosystems from exhibiting significant responses to elevated atmospheric CO₂ (refs 7, 10, 34, 50).

Experiments designed to assess the effect of elevated CO₂ on tussock tundra ecosystems (dominated by *Eriophorum vaginatum*) were conducted at Toolik Lake, Alaska (68° 38' N, 149° 35' W) between 1983 and 1987^{28,41,42,51}. This study was the first *in situ* manipulation of the effects of elevated CO₂ on a natural, unmanaged ecosystem. This fact, and the potential importance of the findings, caused the authors to delay publication of the results until background documents were available in the open literature including the system description²⁸, contemporary net ecosystem CO₂ fluxes⁴¹ and leaf-level photosynthesis responses⁵².

Automated, CO₂- and temperature-controlled greenhouses²⁸ were used to provide *E. vaginatum*-dominated tussock tundra ecosystems with ambient and elevated CO₂ concentrations during the 1983–85 growing seasons at Toolik Lake. The system consisted of 12 chambers that were 1.2 m square and ~0.5 m tall. The experiment included treatments with three replicates each: ambient CO₂ concentration (340 p.p.m.), elevated CO₂ concentration (680 p.p.m.), and elevated CO₂ concentration plus elevated temperature (680 p.p.m. + 4 °C). Three chamberless plots were used as controls. Net CO₂ flux was measured every 3 minutes, and summed and recorded every 6 minutes. Net seasonal flux was determined as the integral of the seasonal daily fluxes.

Tussock tundra exposed to elevated CO₂ and ambient temperatures exhibited a loss of photosynthetic capacity, whereas plots exposed to both elevated CO₂ and elevated temperature retained the enhanced photosynthetic capacity during the 3-year experiment. Ambient plots were net sources of CO₂ to the

atmosphere during all 3 years (Fig. 1). This result has been recently supported in other tussock tundra sites, as well as wet sedge ecosystems on the north slope of Alaska^{41,53,54}. Plots exposed to elevated CO₂ were net CO₂ sinks during the first 2 years of exposure; but during the third year of the experiment, such plots were net sources of CO₂ of ~1 g C m⁻² d⁻¹ (Fig. 1).

Despite rapid (within 3 weeks) leaf-level photosynthetic adjustment of the dominant species *E. vaginatum* to elevated CO₂ (ref. 52), the CO₂ fertilization effect during the first year of exposure resulted in significantly greater net ecosystem CO₂ accumulation (Fig. 2). In the first year of treatment, daily net ecosystem CO₂ flux at 680 p.p.m. was stimulated, and positive, from the start of the experiment until late August (Fig. 3). The increase in CO₂ incorporation at the ecosystem level was due to the presence of species that 'down-regulated' photosynthesis or leaf area (for example *Ledum palustre* and *Betula nana*; W.C.O., unpublished data) more slowly than was the case for *E. vaginatum*. Plots exposed to 680 p.p.m. CO₂ accumulated 6 g C m⁻² d⁻¹ more than ambient plots in the first year. However, this fertilization effect decreased in the second year and virtually disappeared in the third year of the experiment. During the second year of CO₂ fertilization, patches exposed to elevated CO₂ accumulated ~4 g C m⁻² d⁻¹ more than ambient plots, but by the third year, the seasonal CO₂ fertilization effect at 680 p.p.m. CO₂ disappeared entirely (Fig. 2). In the third year of treatment, daily fluxes at 680 p.p.m. CO₂ were similar to those at an ambient CO₂ concentration of 340 p.p.m. (Fig. 3).

Plots that were exposed to both elevated CO₂ and elevated temperature were net sinks for atmospheric CO₂ throughout the 3 years of continuous growing-season experimental manipulation (Fig. 4). Presumably elevated temperatures coupled with absolute air humidities and soil water tables enhanced rates of mineralization and nutrient supply and increased potential sink activity, thereby resulting in prolonged stimulation of photosynthesis. Continued enhancement of photosynthesis by elevated CO₂ should require that nutrients are available to support the use of the increased carbohydrate supply in growth or storage^{8,9,16,34}. Plant growth and photosynthesis are generally limited by nutrient availability in Arctic ecosystems^{55–57}. However, fertilization usually acts to stimulate tissue production rather than to increase leaf photosynthesis^{55,56,58}. Carbohydrate supply may actually exceed plant need in Arctic species⁵².

Carbohydrate sufficiency in *E. vaginatum* and many other Arctic plants^{34,42,52} may explain the rapid ecosystem-level homeostatic adjustment to elevated CO₂ observed here. If carbohydrate use is limited primarily by resource availability, then the sinks for additional carbohydrate produced under conditions of elevated CO₂ will be limited as well^{34,52}. Without adequate sinks,

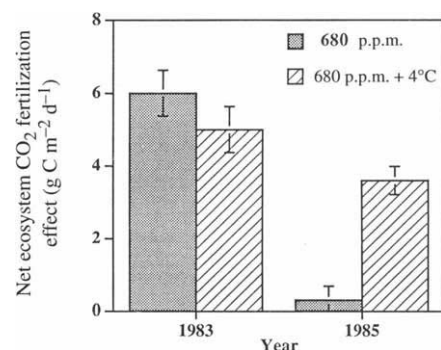


FIG. 4 The CO₂ fertilization effect coupled with a 4 °C increase in ambient temperature on net ecosystem CO₂ flux during the first and last year of the 3-year *in situ* elevated CO₂ experiment at Toolik Lake. Shown is the increase in net ecosystem CO₂ flux at 680 p.p.m. CO₂, and at 680 p.p.m. CO₂ and +4 °C, compared to ambient (340 p.p.m.) CO₂ and temperature. Data are means ±1 standard error; n=3 chambers per treatment.

accumulated carbohydrates may feed back to reduce the amount of Rubisco with a concomitant reduction in photosynthetic potential^{25,52}.

Northern-latitude warming may lead to greater thaw depth and drainage of upper soil layers^{34,41}, which would act to enhance soil aeration, decomposition and mineralization^{40,59,60}. Increased nutrient availability may have stimulated sink activity and strength in tussock plots exposed to elevated temperature^{61,63} allowing for the use of excess carbohydrate reserves, and prolonged photosynthetic response to elevated CO₂ (refs 42, 52).

Increased temperature may also directly increase sink strength and activity, as respiration and growth tend to increase at moderately high temperatures in some Arctic species^{56,63,64}. Hence, increased temperatures predicted for northern-latitude ecosystems may directly or indirectly stimulate ecosystem sink strength.

These results indicate that initial stimulation to tussock tundra ecosystems by elevated CO₂ is transient (with homeostatic adjustment) at 680 p.p.m. CO₂, occurring within 3 years of exposure to elevated CO₂. Under current ambient conditions, whole ecosystem respiration is occurring at a greater rate than is ecosystem photosynthesis. Currently high ecosystem respiration rates are most likely due to decreases in site waterlogging^{41,53,54} following recently reported temperature increases for northern-latitude ecosystems^{44,48}. A concomitant increase in nutrient mineralization should be realized from enhanced soil decomposition⁶⁵, which should lead directly to greater plant growth^{55,56} and greater ecosystem sink strength for CO₂ (refs 10, 34). Genetic constraints to plant growth, however, may limit the ecosystem response to enhanced nutrient availability^{11,14,66}. The potential increase in gross ecosystem productivity, at least in the short term, may not be enough to counteract the increase in whole system respiration.

There are many uncertainties in predicting the long-term effect of global change on Arctic ecosystems. The new equilibrium level of ecosystem productivity, and the resulting carbon balance, will depend on a variety of factors such as plant genetic constraints, plant competition, resource availability and site water balance^{16,34,41,52,56,57,67,69}. Over longer time intervals invasion of shrub or tree species may act to increase above-ground carbon storage^{34,38,41}. There is currently no evidence that these mechanisms are acting, or will act quickly enough to offset a large net efflux of CO₂ to the atmosphere; it seems likely that significant amounts of carbon will be lost before other factors (including altered vegetation composition) result in re-incorporation of this lost carbon back into Arctic ecosystems or their replacement ecosystems⁷⁰.

These results are the first field evidence for complete homeostatic adjustment of CO₂ flux (in a native ecosystem), to elevated atmospheric CO₂, and support conclusions from a variety of sources. This other work includes phytotron experiments on Arctic plants¹¹, Arctic ecosystem microcosms^{68,69} and non-Arctic plants^{17,71}, greenhouse experiments on a simulated tropical ecosystem⁶, and open-top field chamber experiments on grasses⁷² and pine trees²⁷. These studies all showed little or no 'CO₂ fertilization effect' on plants after some brief period of adjustment. These results sound several cautionary notes for those who feel that elevated atmospheric CO₂ will necessarily lead to increased net ecosystem carbon sequestration^{2,73,77}. Clearly, other resources and factors besides CO₂ can limit ecosystem productivity, and therefore potential responses to elevated CO₂. At the same time, CO₂-related climate change can cause a potentially very large release of biospheric CO₂ from terrestrial ecosystems to the atmosphere^{41,53,54}.

Experiments and observations indicate that warmer temperatures with no change in soil moisture increases can cause a rise in net ecosystem CO₂ uptake^{34,38}, while warmer and drier conditions may cause a net loss of CO₂ from the ecosystem to the atmosphere^{41,53,54}. Knowledge of the effects of interaction of

various environmental factors, including elevated CO₂, will be necessary to predict future carbon sequestration and loss by terrestrial ecosystems.

The manipulations reported here were conducted for only three growing seasons. Longer-term experiments or observations are needed to rule out ecosystem response to elevated CO₂ over longer time intervals. This need could be accommodated by long-term field manipulations, and by field observations of natural CO₂ springs, where plants and ecosystems have experienced long-term exposure (perhaps millennia) to elevated CO₂ (refs 77, 78 and W.C.O., unpublished data). Under these conditions very long-term exposure effects at the ecosystem level, including effects on carbon sequestration, can be evaluated. □

Received 28 February; accepted 19 July 1994.

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ACKNOWLEDGEMENTS. We thank J. Jaeger for field assistance, D. Billings for comments on the manuscript, and J. Stock, K. Turner, V. Nosov, and T. Gilmanov for assistance in preparing the manuscript and figures. Accommodation and support provided by the University of Alaska Institute of Arctic Biology at Toolik Lake are gratefully acknowledged. This work was supported by the CO₂ Research Program, Environmental Research Division, Office of Health and Environmental Research of the US Department of Energy.

Climate variations in Europe over the past 140 kyr deduced from rock magnetism

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RAPID shifts in climate during the last glacial are now well documented, particularly from the oxygen isotope records of the two Greenland ice cores GRIP^{1,2} and GISP2³. In the GRIP record^{1,2} these climate events are also seen during the preceding (Eemian) interglacial which may be an analogue for the future climate, warmed by the greenhouse effect. But these shifts are not found in the Eemian section of the GISP2 core³, casting doubt on whether the rapid shifts in the GRIP oxygen isotope record really do represent a climate signal. Here we present magnetic susceptibility, pollen and organic carbon records from maar lake deposits in the Massif Central, France. These data provide an independent record of past climate and we find that they correlate well with the ice-core records during the last glacial. During the Eemian, two rapid cooling events seen in our record also correlate with those seen in the GRIP ice core, supporting the idea that rapid climate change did occur in the Eemian interglacial and demonstrating that it extended to continental Europe.

In sedimentary deposits, the ferrimagnetic fraction originates from detrital inputs and from authigenic or bacterial production⁴; it is diluted in a mineral or biological diamagnetic matrix and is affected by dissolution⁵. Enhancement and impoverishment processes are influenced by environmental and climate conditions prevailing over the sediment source areas. Magnetic susceptibility is a quick, cheap and non-destructive measure of relative concentrations of magnetic oxides (such as magnetite (Fe₃O₄) and titanomagnetite (Fe_{3-x}Ti_xO₄)) and has already contributed to global-change reconstructions from marine^{6,7} and loess-palaeosol sequences^{8,9}.

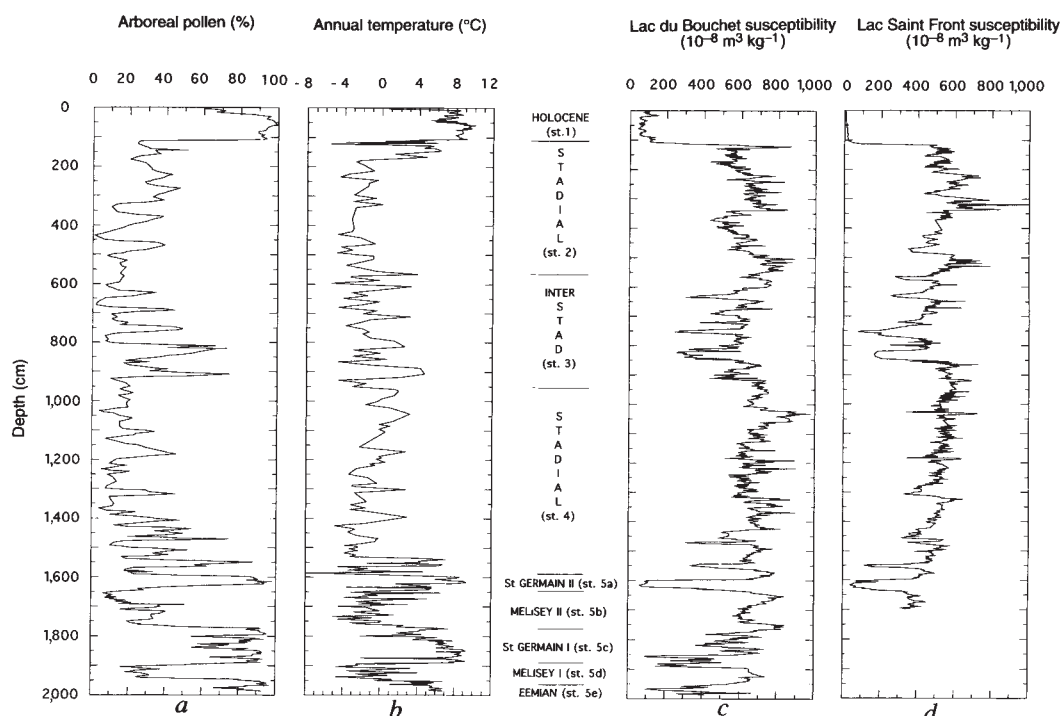
Here we discuss sedimentary sequences deposited in maars (phreatomagmatic explosion craters) of the Massif Central, France. Their topographic position and great initial depths favoured a slow but continuous sedimentation over long periods during late Quaternary times. Under cold climate, freeze-thaw alternations stimulated the erosion of the volcanic rocks and the deposition of clastic sediments. Under temperate climate, vegetation and soil development enhanced the organic sedimentation, favouring dilution and dissolution of the magnetic fraction. The magnetic susceptibility is thus strongly sensitive to variations of the local climate and constitutes an accurate proxy record, particularly useful when palaeobiological or organic geochemical information loses some degree of significance (that is, under the coldest conditions).

Lac du Bouchet and Lac St Front lie 40 km apart in the Velay region (45° N, 3° E) at 1,208 and 1,300 m altitude. They are respectively oligotrophic and eutrophic lakes filling subcircular craters less than 2 km wide, cut through Hercynian granites and Quaternary volcanic formations. Several generations of cores have been obtained since 1982; the last provided a 50-m sequence at Lac du Bouchet¹⁰ and a 65-m sequence at Lac St Front. The upper halves of these sequences covering the last climate cycle are treated here. Magnetic susceptibility was initially used as an independent parameter to correlate parallel records of geomagnetic palaeosecular variation^{11,16}. Hysteresis cycles, thermomagnetic curves, scanning electron microscope observations and X-ray energy-dispersive spectroscopy microprobe analyses established that euhedral grains of detrital titanomagnetites, 0.1–100 µm in size, are the carriers of the magnetic signal^{14,16}.

The average susceptibility record of Lac du Bouchet constructed from a first generation of 12 parallel cores (6–20 m long)^{11,14} was correlated with the Lac St Front record on the basis of pollen and organic carbon markers (Fig. 1). Granulometry, heavy mineral determination, clay mineralogy¹⁷, organic geochemistry^{17,19} and diatom analyses²⁰ independently supplied information on sedimentary environments. The two endmember sediment types are: (1) silty clays supplied by cryoclastic erosion of volcanic rocks under cold climate (susceptibility >400 × 10⁻⁸ m³ kg⁻¹); (2) organic gyttjas formed under temperate climate by autochthonous organic matter and soil degradation products (susceptibility <200 × 10⁻⁸ m³ kg⁻¹). Arboreal pollen percentage^{21,22} and temperature variations reconstructed by transfer functions on pollen assemblages²³ provide a local climate record comparable with the susceptibility record (Fig. 1a–c). A strong negative correlation (−0.8 < r < −0.7) is due to the simultaneity of large amplitude changes recorded at boundaries of climate events. It confirms the climatic significance of the susceptibility record deduced from the simple model introduced above.

The susceptibility record along its original timescale (see Fig. 2 for the depth-to-time transformation) was compared with the most detailed continental climate record available for the last climate cycle: the δ¹⁸O record from the GRIP ice core (Summit, Greenland)^{1,2} (Fig. 3). Susceptibility features and δ¹⁸O cycles (termed Dansgaard-Oeschger events²) present the same wavelengths and similar general shapes. Considering the uncertainties of the chronologies (see refs 24, 25 and Fig. 2) we felt justified in performing slight realignments, in order to improve the corre-

FIG. 1 Vegetational proxy records of Lac du Bouchet: arboreal pollen percentage^{21,22} and annual temperature²³ (present-day temperature, 7 °C) compared with susceptibility records of Lac du Bouchet and Lac Saint Front plotted on a common depth scale.



lation between the two records. Our chronology can be slightly modified according to the following: (1) radiocarbon ages between 12 and 30 kyr before present (BP) are few kyr too young, according to ^{14}C calibration with U/Th datings on corals²⁶; (2) before 30 kyr BP the distribution of our radiocarbon population is rather widespread; (3) the main constraint on our chronological reconstruction before 70 kyr is the identification of vegetational events in western and northern Europe^{21,22,27} to $\delta^{18}\text{O}$ substages²⁸. The result of matching susceptibility peaks with $\delta^{18}\text{O}$ features (maximum distortion is 5 kyr) is a significant correlation ($r = -0.76$) (Fig. 3b, c), suggesting that climate changes over western Europe and Greenland might have been contemporary throughout the last 120 kyr. Correlations between maxima of *Neogloboquadrina pachyderma*, Heinrich layers (H0 to H6), at Deep Sea Drilling Project (DSDP) Site 609 and core VEMA23-81, and Dansgaard-Oeschger events in the GRIP record suggest that cooling episodes of Atlantic surface waters linked with the surges of the Laurentide ice sheet occurred at the same time as cooling episodes of the air masses over Greenland²⁹. High concentrations of calcium and dust in the GISP2 record, correlated with Heinrich events, further document a strong relationship between polar atmospheric circulation and sea ice cover³⁰. Independently, a possible relation between Heinrich events at DSDP Site 609 and *Pinus* events in the Lake Tulane sequence (Florida)³¹ suggested that lower-latitude regions in North America were under the same influence. A link between susceptibility cycles, Dansgaard-Oeschger events and Heinrich events (Fig. 3 and ref. 29) would further suggest that over western Europe, the climate was strongly influenced by sea surface temperatures and atmospheric conditions over North Atlantic and Greenland areas, through the past 120 kyr.

These geographical linkages suggest that it might be possible to verify on middle latitude continental sites, the hypothesis of climate variability during the last interglacial as deduced from the GRIP record¹ and weakened by the GISP2 record³. Climate oscillations during the interglacial-glacial transition have already been reported from Les Echets and La Grande Pile³²⁻³⁴.

In the 20-m susceptibility record from Lac du Bouchet (Figs 1, 3), a rebound to high susceptibility values appeared at the

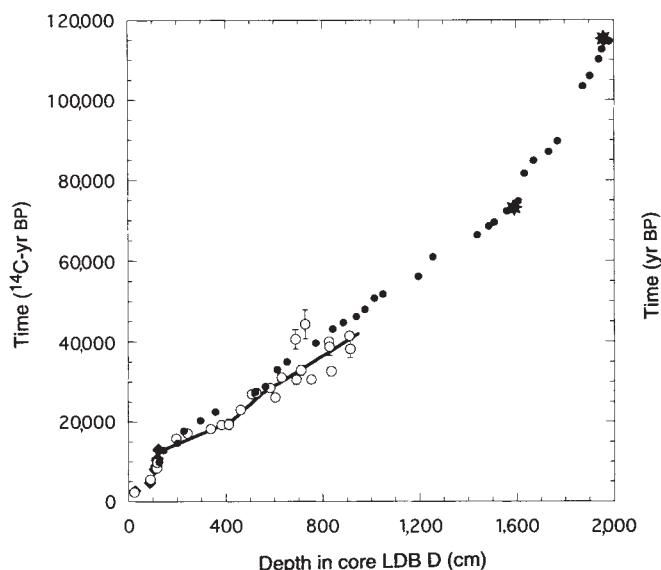
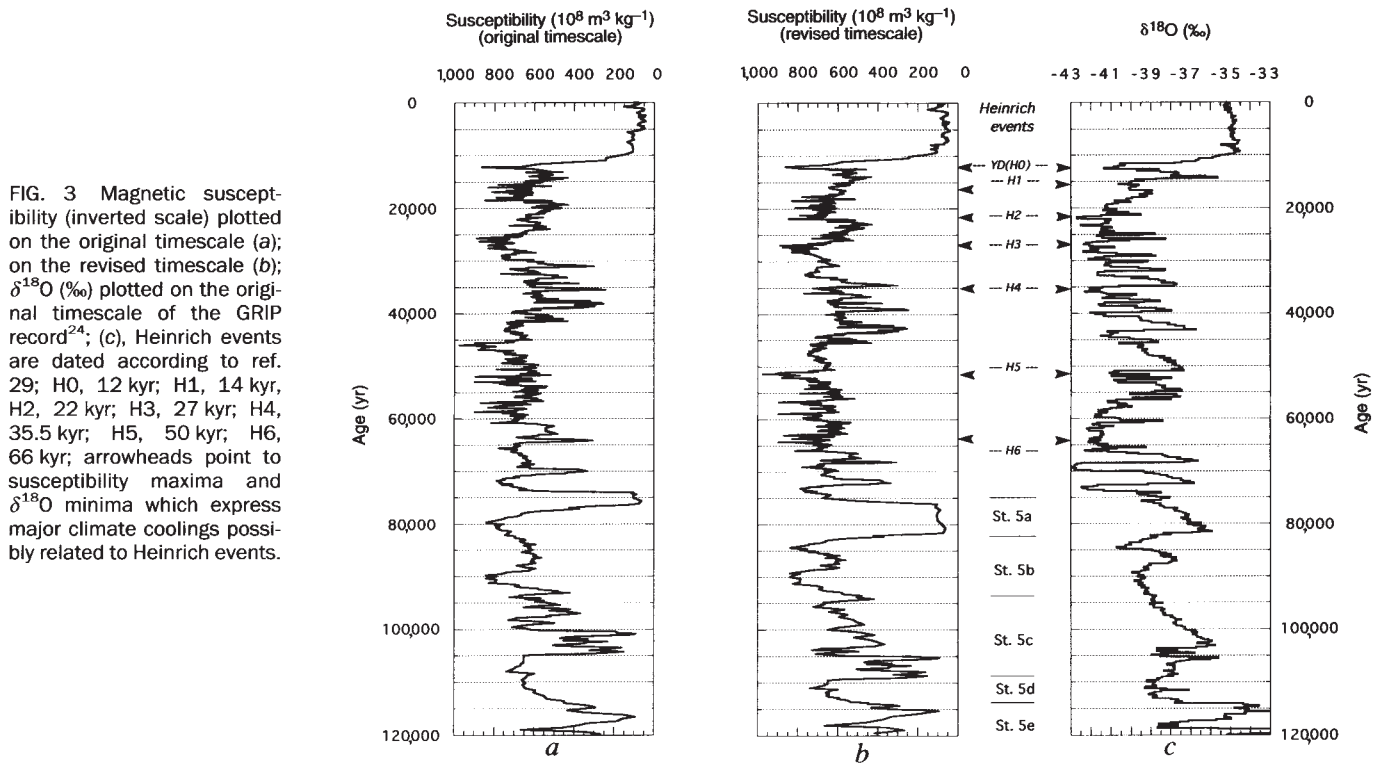


FIG. 2 Depth-to-time transformation based on 25 radiocarbon ages (empty circles) completed by vegetation markers (black diamonds) dated by multiple ^{14}C determinations in other records of the same region. For the interval 10–40 kyr BP, three weighted regression lines (lines) were calculated from 22 radiocarbon ages distributed between 1.2 and 9 m in depth. Before 40 kyr BP, two vegetational markers (stars) were correlated with $\delta^{18}\text{O}$ isotopic stages of the oceanic record²⁸: end of stage 5a, 73 kyr; end of stage 5e, 115 kyr. Susceptibility peaks used for correlation and time scale adjustment are identified by black dots (see also text and Fig. 3).

end of the Eemian; its reality could be checked in the extended sequence of clays and gyttjas collected in the 50-m cores H and I (Fig. 4). The limits of the Eemian layers are unambiguously located by pollen determinations at 20.67 m and 21.60 m in core H, and at 20.40 m and 21.30 m in core I. Lithological changes, susceptibility, organic content and pollen have original signatures that prevent any confusion with later isotope stages (5a to 5d), which have been clearly identified in the overlying sediments



of the same cores. A lower dark-brown gyttja unit, characterized by weak susceptibilities (down to $100 \times 10^{-8} \text{ m}^3 \text{ kg}^{-1}$) and high organic content (up to 9%) contains the highest concentration of arboreal pollen (*Quercus* and *Corylus*); this facies can be interpreted as a first warm stage ending with the expansion of *Carpinus* and the recession of *Quercus*. Above, a 50 cm thick brown silty-clayey unit with strong susceptibilities (up to $800 \times 10^{-8} \text{ m}^3 \text{ kg}^{-1}$) and low organic content (down to 3%) containing the expansion of *Abies*, *Picea*, *Poaceae* and other non-arboreal taxa, documents the increase of erosional processes and

the development of an open subalpine vegetation under colder and wetter conditions. This cold episode is divided by a slightly warmer event documented in a thin dark clayey layer by a decrease of susceptibility, a slight increase of *Quercus* and a recession of grasses. The upper dark gyttja layer carrying weak susceptibilities and moderate carbon contents is characterized by the dominance of *Pinus*, with presence of *Picea*, and *Quercus*, suggesting a drier and slightly warmer climate. Finally, brown clays mark the transition to the Melisey stadial (stage 5d) characterized by an increase of susceptibility and a decrease of car-

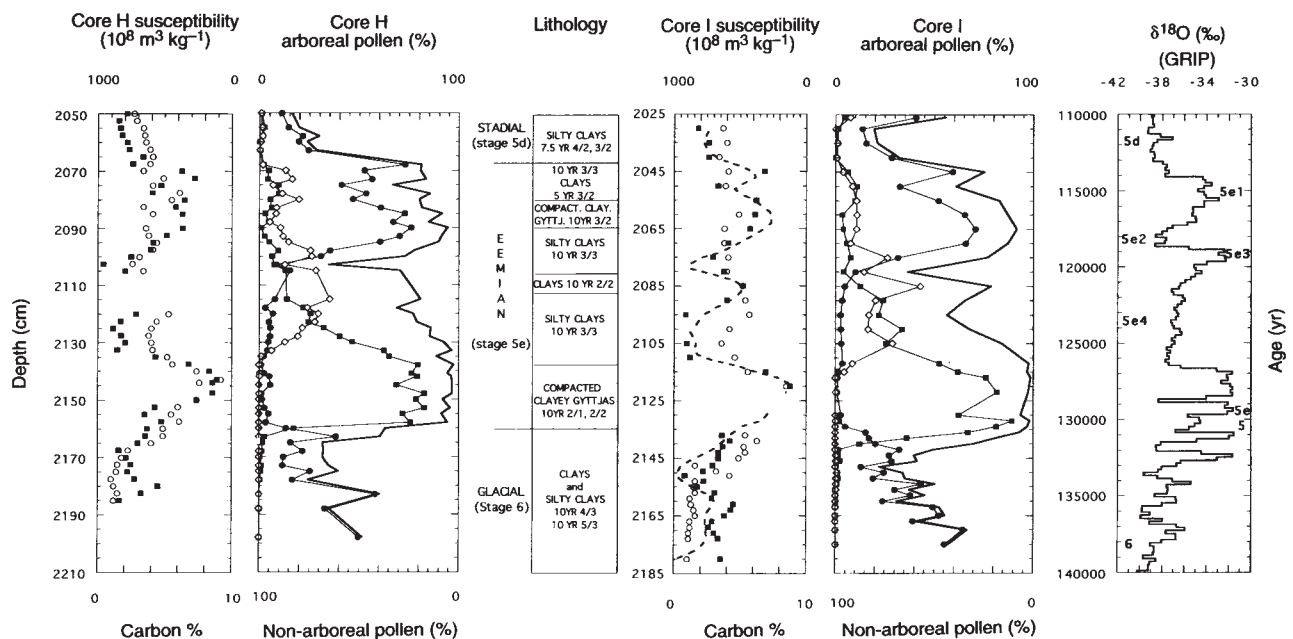


FIG. 4 The Eemian record of the Lac du Bouchet according to susceptibility (inverted scale) (filled squares for specimens and dotted line for whole-core measurements), organic carbon (empty circles) and pollen (filled squares, *Quercus* + *Corylus* + *Carpinus*; empty diamond, *Picea*;

solid dots, *Pinus*; unbroken line, non-arboreal pollen). The lithology is drawn from both cores. Pollen percentages are all plotted on a 100% scale (inverted scale for non-arboreal pollen). The GRIP record is presented on the right for comparison.

bon content. *Pinus* and other arboreal taxa recede, while non-arboreal taxa increase, reaching a maximum extension in the brown silty clays of the Melisey I substage (5d).

Thus the susceptibility, organic carbon and pollen record of Eemian at Lac du Bouchet documents five climate oscillations that might be related with substages 5e5 to 5e1 described in the GRIP record¹. We emphasize that the two cooling phases occurring after the first optimum are not followed by a full and lasting return to a temperate climate. This agrees with the gradual attenuation of the amplitude of $\delta^{18}\text{O}$ maxima 5e5, 5e3 and 5e1 in the GRIP record and could suggest a long and complex interglacial-glacial transition. At this stage however, these results strongly support the hypothesis of climate variability during the Eemian interglacial. □

Received 9 March; accepted 19 August 1994.

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ACKNOWLEDGEMENTS. We thank C. Hillaire-Marcel, A. De Vernal and T. Johnson for discussion and P. Guenet for coring engineering. This work was supported by the EU (Geomaars and Euromaars projects), the French Institut National des Sciences de l'Univers and by the CNRS.

Constraints on the melting temperature of the lower mantle from high-pressure experiments on MgO and magnesiowüstite

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THE melting temperatures of minerals in the Mg–Fe–Si–O-system play a fundamental role in the chemical differentiation, rheology and geodynamics of the Earth's lower mantle. We have previously shown¹ that the melting curve of (Mg, Fe)SiO₃-perovskite—the dominant mineral in the lower mantle—is extremely steep, implying melting temperatures at the bottom of the lower mantle in excess of 7,000 K. The large difference between actual mantle temperatures and the melting temperature inferred from our experiments suggests that the viscosity of the lower mantle is much larger than that typically used in convection models². Theoretical estimates of the melting temperature of MgO (refs 3–5) suggest even higher melting temperatures for (Mg, Fe)O-magnesiowüstite, the second most abundant mineral in the lower mantle. We show here, however, that the melting curves of these two minerals are flat compared to the perovskite melting curve, thus lowering the upper bounds for the solidus in the lowermost mantle to about 5,000 K. This reduces the estimate of the viscosity to more realistic levels but still rules out large-scale melting in the lower mantle. Because magnesiowüstite is slightly more dense than Mg–Fe–Si-perovskite due to iron partitioning, chemical segregation in the lower mantle cannot be excluded in regions where the local temperature exceeds the solidus.

The melting temperature of the lower mantle is assumed to be near the eutectic in the phase diagram MgO–FeO–SiO₂, representing most likely ~90% of the lower-mantle chemistry. It has been suggested previously that (Mg, Fe)SiO₃-perovskite lies

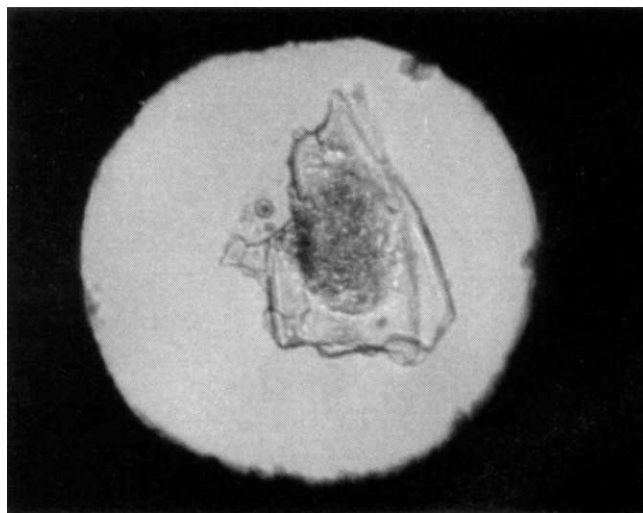


FIG. 1 Sample of single crystal of MgO in an argon pressure medium in the diamond cell after a melting experiment at 315 kbar. Sample dimensions are $\sim 100 \times 100 \times 15 \mu\text{m}$. The molten part of the sample recrystallized on cooling.

near the eutectic composition⁶, but this requires higher melting temperatures for the endmembers MgO and SiO₂ than those previously measured for perovskite¹. The pressure dependence of the melting temperature of MgO has never been measured, and the melting curve of stishovite, the high pressure phase of SiO₂, is poorly constrained by the scarce shock-wave data^{7,8}. Here we present the first experimental data on the pressure dependence of the melting temperatures of MgO and (Mg_{0.85}Fe_{0.15})O-magnesiowüstite using identical laser-heating techniques as in our previous work on (Mg, Fe)SiO₃-perovskite¹ and iron⁹, respectively. The pressure dependence of the melting of MgO has not been measured previously because of its high initial melting temperature of 3,063 K (ref. 10) which prevented the use of thermocouples and conventional high-pressure techniques. Laser-heating experiments (using a yttrium aluminium garnet (YAG) laser) in diamond cells failed because of the low

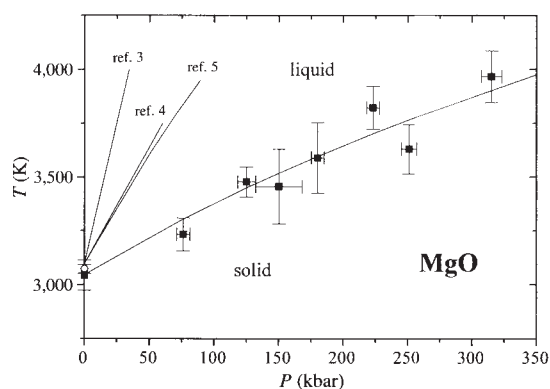


FIG. 2 Melting temperatures of MgO (squares) as a function of pressure. Our value at 1 atm differs from the latest literature value¹⁰ (circle) by ~ 20 K. Pressures were measured from smaller ruby chips distributed within the pressure cell. Contact between ruby and sample was avoided. The pressure error bars represent uncertainties in the thermal pressure and in the pressure gradients. Temperatures were measured directly from areas of $3\ \mu\text{m}$ diameter by fitting a Planck radiation function to the emission spectra from 500 to 800 nm with both temperature and emissivity as free parameters. The emissivity was assumed to be independent of wavelength. The largest uncertainty associated with this assumption is estimated as about ± 300 K at the highest temperatures of this study. The temperature error bars indicate the variation of at least 5 measurements of the onset of melting. The melting curve is a fit of a Lindemann equation to the data. Previous estimates are from thermodynamics^{4,5} and from molecular dynamics³.

absorption of that radiation in MgO. Here we use CO₂-laser heating techniques and thermally insulated samples, and show that previous theoretical estimates on the initial melting slope of MgO are too high, and that its melting curve crosses that of (Mg, Fe)SiO₃-perovskite at ~ 500 kbar. This implies that the MgO-SiO₂ phase diagram changes substantially at higher pressures, and that perovskite is not the low-melting phase in this system.

Details of the laser-heating experiments are described elsewhere¹. The essential features of this experiment are the use of CO₂-laser radiation (which is almost fully absorbed by MgO) and of very thin samples that are surrounded by an inert, thermally insulating argon pressure medium which itself does not absorb laser radiation or emit incandescent light. The small thickness of the sample and a defocused laser beam reduce axial and radial temperature gradients in the sample, respectively. Melting was detected *in situ* by visual observation of large

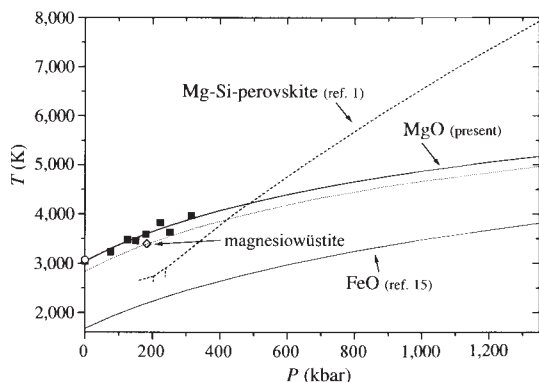


FIG. 3 Extrapolated melting curves in the lower mantle using a Lindemann equation (see text). For MgO, a linear T_m versus compression relation (Kraut-Kennedy)¹³ yields almost identical results. One measurement for (Mg_{0.85}Fe_{0.15})O-magnesiowüstite yields an estimated melting curve which is parallel to the melting curves of MgO and of FeO (ref. 15). The melting curve of (Mg, Fe)SiO₃-perovskite measured previously to 625 kbar (ref. 1) is shown for comparison.

increases in temperature which is due to an increase in the optical absorption of the melt.

Here we report melting temperatures between 1 atm and 315 kbar. The most recent measurement of the 1-atm melting temperature of MgO found in the literature is $3,063 \pm 20$ K (ref. 10). The present measurement taken outside the diamond cell in air yields a melting temperature T_m of $3,040 \pm 100$ K. The melting data are shown in Fig. 2. Melting temperatures were determined by slowly increasing the laser power while recording temperatures in rapid sequence. Melting temperatures reported here are the last temperatures before melting set in. A Lindemann relation¹¹ of the form $d \ln T_m / d \ln \rho = 2(\gamma - 1/3)$, where ρ is the density and γ is the thermal Grüneisen parameter, was fitted to the data and is shown by the solid curve. The densities were calculated from a third-order finite-strain equation of state and the pressure dependence of the Grüneisen parameter was taken¹² as $\gamma/\gamma_0 = (\rho/\rho_0)^{1.3}$. The resulting value for the 1-atm Grüneisen parameter is 1.3, in good agreement with that reported in the literature. The slope of the melting curve at 1 atm is $3.6\ \text{K kbar}^{-1}$. Also shown in Fig. 2 is the estimate by Ohtani⁵, who used a Kraut-Kennedy relationship and an initial slope estimate by Jackson⁴, which was based on elastic shear instability and analogy with LiF. This yielded an initial melting slope of $11\ \text{K kbar}^{-1}$. Other calculations using similarities between the MgO-SiO₂ and the LiF-BeF₂ phase diagrams, and calculations involving the activation volume and activation energy of MgO, yielded similar high values of the melting slope³. The highest melting temperatures were predicted from molecular dynamics calculations³.

Figure 3 shows melting temperatures extrapolated to the pressure of the core-mantle boundary using the Lindemann relation. For MgO this yields melting temperatures at the bottom of the lower mantle of slightly above 5,000 K, provided that there are no phase transitions in this pressure range. A linear T_m -compression relationship¹³ yields nearly identical results. A melting temperature of (Mg_{0.85}Fe_{0.15})O-magnesiowüstite of $3,400 \pm 100$ K was measured in an argon pressure medium at 182 kbar using a yttrium-lithium-fluoride (YLF)-laser (wavelength $\lambda = 1.05\ \mu\text{m}$) and melting criteria described elsewhere⁹. This material was synthesized by Ringwood (Australian National University) and represents the likely composition of magnesiowüstite at lower-mantle conditions¹⁴. The previously measured melting curve of FeO (ref. 15), extrapolated using the Lindemann formula, is nearly parallel to the melting curve of MgO and the melting temperature of magnesiowüstite is in perfect agreement with the melting behaviour of a solid-solution system between MgO and FeO. For comparison we show the recently measured melting curve of (Mg, Fe)SiO₃-perovskite¹. Our estimated melting curves of MgO and of (Mg, Fe)O-magnesiowüstite cross that melting curve at ~ 500 and 430 kbar, respectively. This implies that for most of the lower mantle the upper bound for the lower-mantle solidus is substantially lower than previously assumed¹. The estimate of the eutectic temperature in the MgO-FeO-SiO₂ phase diagram will require further measurements on SiO₂-stishovite and on multi-component systems.

The lower melting temperatures of (Mg, Fe)O-magnesiowüstite as compared to (Mg, Fe)SiO₃-perovskite may have important geophysical and geochemical implications. In a recent study² the effect of the very high melting temperatures obtained for Mg-Si-perovskite on mantle dynamics was investigated using an Arrhenius-type creep law and Weertmann's¹⁶ assumption on the basis of homologous temperatures, T/T_m , where T is the actual mantle temperature and T_m is the melting temperature. This resulted in a very large increase in the estimated viscosity with depth in the lower mantle, suggesting a rather passive role of the lower mantle in large-scale deformation. If the lower mantle contains a substantial amount of magnesiowüstite, however, its low shear strength and the higher homologous temperature may reduce the viscosity substantially.

It has been shown that for a mixture of (Mg, Fe)SiO₃-perovskite and (Mg, Fe)O-magnesiowüstite, iron partitions preferentially into magnesiowüstite at lower-mantle conditions¹⁴. These iron-partitioning measurements are corroborated by qualitative observations on the reactivity of iron with these two phases at *P-T* conditions of the core-mantle boundary (1.35 Mbar, >4,000 K). In these experiments, the contact zone between MgSiO₃ (or MgO) and iron was heated with a YLF-laser; the heated zone was then checked for chemical reactions, using a microscope. Although such experiments are only quantitative, similar experiments using resistively heated iron wires have shown that chemical reactions were always accompanied by a strong discoloration of the surrounding pressure medium. (Reactions were inferred to have occurred on the basis of residual resistance changes in the wire.) Here we find that for dry conditions, where the samples were dried in a vacuum at 120 °C and subsequently flushed with dry argon, no such discoloration was observed in contact zones between molten iron and MgSiO₃ at temperatures exceeding those of the melting temperature of iron. In contrast, large reaction zones between MgO and iron were found, even at temperatures below the melting point of the latter. We do not know the nature of this reaction which may yield non-stoichiometric products, but these experiments demonstrate a drastic difference in the behaviour of these two materials towards iron.

It has been shown (using known compressibilities and systematics of the pressure dependence of thermal expansivity) that for the lowermost mantle, the iron-enriched (Mg, Fe)O-magnesiowüstite phase is denser than the iron-depleted (Mg, Fe)SiO₃-perovskite phase by ~2% (ref. 17). Gravitational segregation of magnesiowüstite can therefore not be ruled out but most likely is ineffective if the phases are subsolidus, well mixed and of small grain size. On the other hand, segregation may occur if temperatures exceed the solidus temperatures of the system and the liquidus phase is iron-rich magnesiowüstite. The average temperature of the lower part of the lower mantle has been estimated to lie between 2,550 and 2,750 K. The thermal gradient across the core-mantle boundary is at least 1,300 K, estimated from melting measurements of pure iron and on iron-oxygen mixtures⁹. Therefore the temperature at the very bottom of the lower mantle in contact with the core is on average ~4,000 K or still ~1,000 K below the melting temperature of magnesiowüstite. Melting of parts of the lower mantle cannot therefore be ruled out in the early history of the Earth, but would now require a substantially lower solidus temperature of the system, or additional heat sources such as frictional heating (D. Yuen, personal communication). Gravitational segregation of iron-enriched magnesiowüstite from Mg-Si-perovskite would inevitably lead to an iron enrichment at the very bottom of the lower mantle, thus providing an alternative explanation for the *D''* region. □

Received 6 June; accepted 2 September 1994.

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ACKNOWLEDGEMENTS. We thank A. Chopelas, D. Yuen and S. Kesson for helpful discussions, and J. M. Brown for a critical review.

Variation in the TNF- α promoter region associated with susceptibility to cerebral malaria

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TUMOUR-necrosis factor- α (TNF- α) is believed to have an important role in the pathogenesis of severe infectious disease¹ and fatal cerebral malaria is associated with high circulating levels of this cytokine^{2,3}. In a large case-control study in Gambian children we find that homozygotes for the *TNF2* allele, a variant of the TNF- α gene promoter region⁴, have a relative risk of 7 for death or severe neurological sequelae due to cerebral malaria. Although the *TNF2* allele is in linkage disequilibrium with several neighbouring HLA alleles, we show that this disease association is independent of HLA class I and class II variation. These data suggest that regulatory polymorphisms of cytokine genes can affect the outcome of severe infection. The maintenance of the *TNF2* allele at a gene frequency of 0.16 in The Gambia implies that the increased risk of cerebral malaria in homozygotes is counterbalanced by some biological advantage.

It is not known why cerebral malaria occurs in only a small proportion of individuals infected with *Plasmodium falciparum*. A critical factor may be the amount of TNF- α that is produced by the host when malarial schizonts rupture⁵. Clinical studies have demonstrated an association between TNF- α levels and disease severity^{2,3,6} and experimental studies have revealed several ways in which excessive TNF- α production could promote cerebral malaria^{7,8}. A prominent feature of this condition is parasite sequestration within cerebral blood vessels, and TNF- α is known to upregulate the expression of host adhesion molecules that mediate the binding of parasites to vascular endothelium⁹. It has also been proposed that high local concentrations of TNF- α may stimulate cerebral endothelium to over-produce nitric oxide, thus causing coma by interfering with neurotransmission¹⁰.

These observations raise the possibility that cerebral malaria is related to the genetic propensity of the host to produce TNF- α . The gene for TNF- α resides within the class III region of the major histocompatibility complex and several studies have shown that individual differences in TNF- α production can be linked to HLA type and other polymorphic markers in this region^{11–13}. This suggests that TNF responsiveness is controlled by variable genetic elements within the MHC but the precise location of these elements has yet to be identified. A polymorphism that may directly affect the regulation of TNF- α is located at –308 nucleotides relative to the transcriptional start site of the gene^{4,14}. There are two allelic forms, referred to as *TNF1* and *TNF2*. Studies of the TNF promoter linked to a chloramphenicol acetyltransferase reporter gene have shown that the *TNF2* allele is associated with higher constitutive and inducible levels of transcription than the *TNF1* allele (ref. 15, and A. G. Wilson and G. W. Duff, personal communication). Because of the possible functional significance of this polymorphism, we investigated its association with cerebral malaria.

Our analysis was based on a large case-control study of malaria in Gambian children (Table 1). Three hundred and seventy-six cases of cerebral malaria were compared with two groups

TABLE 1 *TNF2* allele and homozygote frequencies

(a) Allele frequencies				(b) Homozygote frequencies stratified by ethnic group					
	<i>TNF2</i> homozygotes (%)	<i>TNF2</i> heterozygotes (%)	Sample size		andinka	ola	olof	ula	ther
Mild malaria controls	1.2	29.8	(325)	Mild non-malaria controls	2.0% (151)	0.0% (41)	0.0% (38)	0.0% (35)	1.7% (60)
Mild malaria	1.8	26.8	(332)	Cerebral malaria All cases	3.9% (153)	8.1% (62)	5.1% (59)	2.3% (44)	3.4% (58)
Severe malarial anaemia	1.8	31.5	(111)	Deaths and sequelae	7.0% (43)	15.0% (20)	0.0% (13)	9.1% (11)	8.3% (12)
Cerebral malaria All cases	4.5	26.6	(376)						
Deaths and sequelae	8.1	25.3	(99)						

a, Percentage of children carrying the *TNF2* allele in each clinical category; b, more detailed breakdown of homozygote frequencies by ethnic group. The figures in parentheses indicate sample size. When analysed by Mantel-Haenszel test after stratification by ethnic group, the *TNF2* homozygote frequency was significantly higher in cerebral malaria than in mild malaria or in controls with mild illnesses other than malaria. **Study design.** The details of this case-control study have been described previously²². Children aged 1 to 10 years were enrolled, with the permission of a parent or guardian, at the Royal Victoria Hospital, Banjul, the MRC Clinic, Fajara, and neighbouring health centres during the 1988–1990 rainy seasons. The study was approved by the Gambian Government/MRC Joint Ethical Committee. Cerebral malaria was defined as a Blantyre coma score of ≤ 2 (persisting for >30 min after effective treatment of hypoglycaemia or convulsions) or repeated prolonged seizures (>30 min) in a child with *P. falciparum* parasitaemia and no other apparent cause of fits or coma. Severe malarial anaemia was a haemoglobin <5 g dl⁻¹ in association with asexual *P. falciparum* parasitaemia of $>2,500$ μ l⁻¹. In the table, the cerebral malaria sample includes 45 children who also had severe anaemia. The primary control group, chosen to represent background gene frequencies, were children who presented to clinic with a variety of mild illnesses other than malaria: all were free of parasitaemia and well enough to be treated as outpatients. Around 65% of these 'mild non-malaria' controls had upper respiratory tract infections, mild chest infections, or otitis media; 25% had mild gastroenteritis or other abdominal complaints; and 10% had other minor illnesses. A separate group of 'mild malaria' controls had an uncomplicated febrile illness associated with asexual *P. falciparum* parasitaemia and no other evident cause of fever. The severe malaria group was matched to the two control groups for area of residence around Banjul. The mean age of the cerebral cases was 3.9 yr; mild malaria controls, 3.9 yr; mild non-malaria controls, 2.9 yr; severe malaria anaemia cases, 2.3 yr. Because of the mixed ethnic composition of the study population (45% Mandinka, 15% Jola, 14% Wolof, 11% Fula and 15% other groups) our statistical analyses included stratification for ethnic group. 72 of the children with cerebral malaria died. A further 27 had disabling neurological sequelae when discharged from the ward: the typical clinical features have been previously described²⁷. Deaths and severe neurological sequelae were combined in this analysis because of our previous finding that both of these adverse outcomes are associated with high TNF levels³. **Typing of the *TNF- α* gene.** We typed two allelic forms, referred to as *TNF1* and *TNF2*, that

are identical apart from a single base transition (G to A) at -308 of *TNF- α* ⁴. PCR was used to amplify a 519-bp fragment of the *TNF- α* promoter region, spanning -502 to +17 relative to the transcriptional start site as defined in ref. 28. Around 100 ng genomic DNA extracted from venous blood was added to 25 μ l reaction mixture containing 0.1 μ M of each primer (5'-CAAACACAGGCCTCAGGACTC-3' and 5'-AGGGAGCGTCTGCTGGCTG-3') with 100 μ M of each dNTP, 67 mM Tris-HCl, 16 mM (NH₄)₂SO₄, 2 mM MgCl₂, and 0.01% Tween-20. After heating at 95 °C for 10 min, 1.5 U of *Taq* DNA polymerase was added, followed by 35 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, then a final 10 min at 72 °C. Template-free controls were included in each experiment. The PCR product was denatured with 0.4 M NaOH, dotted onto nylon membrane via a vacuum manifold, and fixed for 1 min in UV light. Allele-specific oligonucleotides (5'-AGGGGCATGGGGACGGG-3' for the *TNF1* allele and 5'-AGGGGCATGAGGACGGG-3' for the *TNF2* allele) were end-labelled with digoxigenin-ddUTP (Boehringer Mannheim) and hybridized to the membranes in 3 M TMAC (tetramethylammonium chloride) solution at 55 °C for 1 h. Excess probe was removed by washing in 2 $\times$ SSPE/0.1% SDS at room temperature and subsequently in 3 M TMAC at 58 °C. The membranes were treated with anti-digoxigenin Fab fragments and, after washing to remove excess antibody, they were incubated with Lumigen PPD (Boehringer Mannheim) and exposed to X-ray film for 15–30 min. Allele type was scored by two independent observers. The accuracy of this method was confirmed by DNA sequencing of the PCR product, as described below, in three *TNF1* homozygotes, three *TNF2* homozygotes, and four heterozygotes. **DNA sequencing.** To explore the possibility that the *TNF2* allele might be linked to some other regulatory polymorphism, we sequenced between -502 and +250 relative to the transcriptional start site of the *TNF- α* gene in 20 individuals who were typed by PCR as *TNF2* homozygotes. The PCR product described above was purified and directly sequenced using the cycle sequencing technique (US Biochemical) with the internal oligonucleotide 5'-TCCTGCATCCTGTCTGGAAG-3' in a labelling reaction which omitted dCTP. In addition, we used the primers 5'-CCGCTGGTTGAATGATTCT-3' and 5'-TGATCCTGAAGAGGAGAGA-3' to amplify a second overlapping PCR product which was cycle sequenced over the region -73 to +250 using the internal oligonucleotide 5'-CCTCCTCTCGCCCCAGGG-3' (omitting dGTP in the labelling reaction). No polymorphisms other than the single base substitution at -308 were detected.

of children recruited at the same clinics, frequency-matched for area of residence. Our primary control group was composed of children who presented to clinic with a variety of mild illnesses other than malaria: these 'mild non-malaria' controls were used to estimate background allele frequencies and relative risks. The second group were children with uncomplicated malaria fever due to *P. falciparum*: these 'mild malaria' patients were used to test the hypothesis that the *TNF2* allele predisposes to cerebral malaria rather than malarial illness in general. A further group of children with severe malarial anaemia but without cerebral complications was also investigated.

To type the polymorphism, we amplified the region by the polymerase chain reaction (PCR) and hybridized with allele-specific oligonucleotides (Table 1). The gene frequency of the *TNF2* allele in this population was 0.16, and its distribution in the control groups was consistent with Hardy-Weinberg equilibrium. Among cases of cerebral malaria there was a significantly higher frequency of *TNF2* homozygotes than in non-malaria controls ($\chi^2=6.5$, $P=0.01$) or mild malaria patients ($\chi^2=4.1$, $P=0.04$). The frequency of *TNF2* homozygotes was even higher in those cases of cerebral malaria who died or developed gross

neurological sequelae: this was analysed by Fisher exact test, because of the small number of homozygotes involved, and found to be significantly different from non-malaria controls ($P=0.001$) or mild malaria patients ($P=0.005$). Cases of severe malarial anaemia without cerebral complications had a similar frequency of *TNF2* homozygotes to the two control groups. Using the mild non-malaria controls to represent the background population and stratifying for ethnic group, we estimate that *TNF2* homozygotes have a relative risk of 4.0 for cerebral malaria (95% confidence interval (CI), 1.2–13.8; $\chi^2=5.4$, $P=0.02$, Mantel-Haenszel test) and of 7.7 for death or sequelae due to cerebral malaria (95% CI, 1.9–30.0; $\chi^2=10.2$, $P=0.001$).

Several features suggest that this polymorphism in the *TNF- α* gene could be a causal predisposing factor for cerebral malaria. The available evidence from transfection studies indicates that the *TNF2* allele acts to increase the transcription of *TNF- α* ¹⁵. This is consistent with our observation that homozygotes for the *TNF2* allele are at increased risk for cerebral malaria, as we have previously found that Gambian children with cerebral malaria have higher TNF- α levels than children with mild malaria³. TNF- α levels are highest in those children who die or develop

TABLE 2 HLA phenotypes associated with the *TNF2* allele

(a) HLA phenotypes			
	TNF2+	TNF2-	
B70+	43	39	OR = 2.7, P_{corr} = 0.0009
B70-	140	345	
B50+	19	8	
B50-	164	376	OR = 5.5, P_{corr} = 0.0005
Cw2+	44	39	
Cw2-	139	345	
DRB1*13.02+	148	234	OR = 1.7, P_{corr} = 0.0005
DRB1*13.02-	295	788	
DRB1*11.01+	117	112	
DRB1*11.01-	326	910	OR = 2.9, P_{corr} = 1.3×10^{-12}
DRB1*13.03+	8	56	
DRB1*13.03-	435	966	
			OR = 0.3, P_{corr} = 0.03

(b) Frequencies of HLA class I types associated with *TNF2*

	Controls ($n = 144$)		Cerebral malaria ($n = 215$)	
	Apparent homozygotes	Heterozygotes	Apparent homozygotes	Heterozygotes
B70	3.5	11.8	0.5	14.0
B50	0.0	2.8	0.5	5.1
Cw2	(4.2)	7.6	(6.0)	11.2

(c) Frequencies of HLA class II types associated with *TNF2*

	Controls ($n = 325$)		Cerebral malaria ($n = 374$)	
	Homozygotes	Heterozygotes	Homozygotes	Heterozygotes
DRB1*13.02	4.6	24.3	1.3	24.1
DRB1*11.01	0.0	14.8	0.0	15.5
DRB1*13.03	0.0	3.4	0.0	5.1

a, Significant associations between the *TNF2* allele and HLA phenotypes that are present at $\geq 2\%$ frequency in this population. Because of the multiple comparisons made, P values were corrected by multiplying by the number of concurrent tests performed, namely 38 class I types and 18 class II types. *TNF2* was not associated with any HLA-A type. HLA-A1, B8 and DR3 were specifically analysed because of their close linkage to *TNF2* in Europeans¹⁴ but they were not associated with *TNF2* in this population. OR, odds ratio. b, c, Frequencies of the *TNF2*-linked class I and class II phenotypes in mild non-malaria controls and cases of cerebral malaria. Homozygote and heterozygote frequencies were analysed separately because we specifically wanted to exclude the possibility that the increased disease susceptibility of *TNF2* homozygotes could be attributed to an HLA association. The HLA alleles that were positively associated with *TNF2* were not associated with increased susceptibility to cerebral malaria, and the negatively-associated HLA allele was not associated with protection. Class I types were analysed by serological testing of fresh or cryopreserved lymphocytes, using 180 well-characterized antisera in a standard microlymphocytotoxicity assay. The apparent homozygote frequencies given in b tend to be overestimates of the true homozygote rate because of the presence of serologically undetected alleles, particularly the values for HLA-C which are shown in parentheses. Class II types were determined by restriction fragment length polymorphisms using *TaqI* digestion with DRB- and DQB-specific probes, supplemented by PCR-oligonucleotide analysis as described elsewhere²⁹. The full range of HLA types identified is reported in ref. 22.

neurological sequelae due to cerebral malaria and it is in this group that we found the highest frequency of *TNF2* homozygotes. The lack of an association with severe malarial anaemia is consistent with the view that acute excess of TNF- α production is specifically involved in the pathogenesis of cerebral malaria, rather than severe malaria in general¹⁶.

We recognize that the *TNF2* allele could be associated with some other polymorphism affecting TNF- α regulation, although only one other polymorphism in the TNF- α gene has so far been described, at -238 (ref. 17). We sequenced the region of the TNF- α gene that is most likely to be involved in transcriptional regulation^{18, 20} in 20 individuals who were typed by PCR as

TABLE 3 *TNF2* homozygote frequencies allowing for HLA-B53

	HLA-B53 positive <i>TNF2</i>		HLA-B53 negative <i>TNF2</i>	
	homozygotes	homozygotes	homozygotes	homozygotes
	n	%	n	%
Mild non-malaria	91	2.2	233	0.9
Mild malaria	80	0.0	251	2.4
Cerebral malaria				
All cases	59	6.8	308	3.9
Deaths and sequelae	20	10.0	73	6.8

Analysis of possible interactions between *TNF2* and HLA-B53, which is associated with protection from severe malaria in this population²². Typing of 636 Gambian children without malaria showed that frequency of the *TNF2* phenotype was slightly lower in HLA-B53 positive than HLA-B53 negative individuals, but this was not statistically significant (42/148 vs 152/488; OR = 0.88, $P = 0.52$), that is, there was no evidence of a strong negative association between *TNF2* and HLA-B53. The above data show the relationship between *TNF2* homozygote frequency and disease severity, after stratifying for HLA-B53 phenotype. When these stratified data were analysed by Mantel-Haenszel test, *TNF2* homozygotes had a relative risk (compared to mild non-malaria controls) of 4.1 for cerebral malaria ($\chi^2 = 5.7$, $P = 0.02$) and 7.0 for death or sequelae due to cerebral malaria ($\chi^2 = 9.3$, $P = 0.002$), showing that the *TNF2* association is independent of HLA-B53. HLA-B53 antigen types were determined either serologically (as described in Table 2) or by allele-group-specific PCR amplification and oligonucleotide hybridization as previously described³⁰.

TNF2 homozygotes (Table 1). This sequence (-502 to +250) included much of the promoter as well as the 5' untranslated region. No polymorphisms were found other than the single base substitution at -308.

As the TNF- α gene is located at ~ 250 kb centromeric of *HLA-B* and 1 Mb telomeric of *HLA-DR*²¹, we considered the possibility that this association could be due to linkage disequilibrium with neighbouring HLA class I or class II antigens. We found that HLA-B70, B50, Cw2, DRB1*13.02 and DRB1*11.01 were associated with the *TNF2* allele in our study population, yet none of these HLA types was associated with susceptibility to cerebral malaria (Table 2). Conversely, DRB1*13.03 was negatively associated with *TNF2* but was not associated with protection.

We specifically analysed the relationship of *TNF2* with HLA-B53, which is associated with resistance to severe malaria in Gambian children²². In 636 children without malaria we found only a weak negative association between these two alleles (which was not statistically significant), and a Mantel-Haenszel test confirmed that the association of *TNF2* with cerebral malaria is independent of HLA-B53 status (Table 3). Several lines of evidence indicate that these two neighbouring alleles affect malaria outcome through entirely different mechanisms. The protective effect of HLA-B53 seems to be equally strong for homozygotes and heterozygotes²², whereas the *TNF2* association is confined to homozygotes. HLA-B53 is protective against both cerebral malaria and severe malaria anaemia²², whereas *TNF2* is only associated with cerebral malaria. Excessive TNF production is thought to have a specific role in the pathogenesis of cerebral malaria^{7, 10}, while HLA-B53 probably acts by promoting an effective cytotoxic T-cell response against the parasite in the pre-erythrocytic stage of infection²³.

Malaria has been a powerful agent for the selection of genetic polymorphisms which protect the host against severe complications of the infection. The *TNF2* allele exists at a gene frequency 0.16 in The Gambia despite its association with cerebral malaria, implying that the disadvantage for *TNF2* homozygotes is counterbalanced by some biological advantage. This may well be in relation to host defence, as TNF- α promotes a variety of antimicrobial mechanisms in malaria and other infections^{16, 24, 26}. It is possible that the *TNF2* allele is maintained because hetero-

zygotes possess an optimal level of TNF- α response against a broad range of infections; alternatively it is possible that *TNF2* homozygotes are themselves protected against life-threatening conditions other than cerebral malaria. □

Received 17 June; accepted 18 August 1994.

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ACKNOWLEDGEMENTS. We are grateful to the children, their families, and the medical and nursing staff who made this study possible, and particularly D. Brewster who provided invaluable support. Special thanks are due to L. Bayo, L. Manneh, I. Sambou, N. Anstey and P. Twumasi for help with sample collection, and to G. Duff, G. Wilson and S. Bennett for discussions. The work received funding from the MRC and the Wellcome Trust. W.M. is a Wellcome Advanced Training Fellow and D.K. is an MRC Senior Clinical Fellow.

Selective suppression of the magnocellular visual pathway during saccadic eye movements

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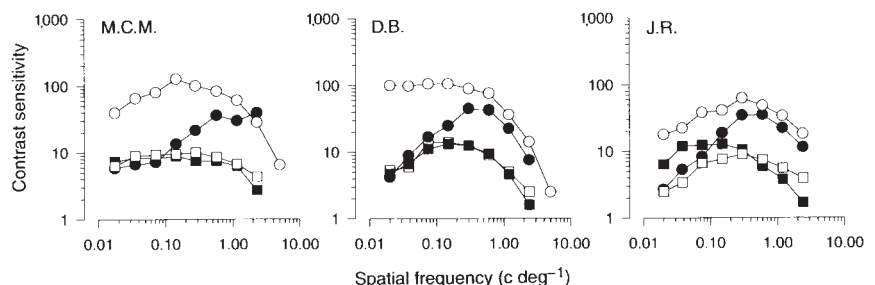
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VISUAL scientists have long sought to explain why the world remains stable during saccades, the ballistic eye-movements that continually displace the retinal image at fast but resolvable¹ velocities. An early suggestion was that vision may be actively suppressed during saccades², but experimental support has been variable^{3–5}. Here we present evidence that saccadic suppression

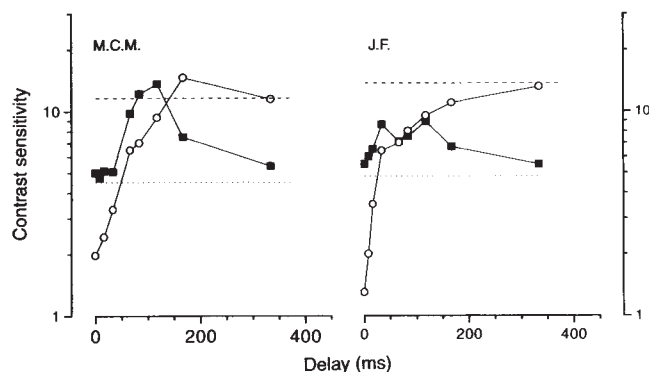
does occur, but that it is selective for patterns modulated in luminance at low spatial frequencies. Patterns of higher spatial frequency, and equiluminant patterns (modulated only in colour) at all spatial frequencies were not suppressed during saccades, but actually enhanced. The selectivity of the suppression suggests that it is confined to the colour-blind magnocellular stream (which provides the dominant input to motion centres and areas involved with attention), where it could dull the otherwise disturbing sense of fast low-spatial-frequency image motion. Masking studies suggest that the suppression precedes the site of contrast masking and may therefore occur early in visual processing, possibly as early as the lateral geniculate nucleus.

We measured contrast sensitivity to horizontal gratings of various spatial frequencies, briefly presented during large voluntary horizontal saccades. As the saccades ran parallel to the bars of the gratings, they produced no effective image motion. The gratings were modulated sinusoidally either in luminance (yellow–black) or chromaticity (equiluminant red–green). Contrast sensitivities for these stimuli are shown by the filled symbols of Fig. 1, together with sensitivities for the same stimuli viewed with the eyes stationary (open symbols). For stimuli modulated in luminance (circles), there was strong suppression of sensitivity during saccades at low spatial frequencies, whereas sensitivity at high spatial frequencies was virtually unchanged

FIG. 1 Contrast sensitivity (inverse of contrast at threshold) for detecting briefly presented (17 ms) horizontal gratings of variable spatial frequency, modulated either in luminance (circles) or colour (squares). Measurements were made both in free viewing conditions (open symbols) and during large (40°) horizontal saccades (filled symbols). Stimuli were generated by a VSG colour framestore (Cambridge Research Systems) under computer control, and displayed on the face of a Mitsubishi colour monitor at 120 Hz, 600 lines per frame at a mean luminance of 10 cd m⁻². The screen was 40 by 30 cm, subtending 67° by 53° at 30 cm viewing distance. It was surrounded by 1.5 × 1 m white card, floodlit by two lateral projectors to a similar mean luminance and chromaticity as the screen (thresholds were unaffected by turning off the floodlight). In the chromatic condition, the gratings were modulated in chromaticity (red–green), equiluminant by standard flicker–photometry criteria (see ref. 27 for more details of stimuli). The equiluminant point measured during saccades was the same as that in free viewing. Voluntary saccadic eye movements were recorded with electrodes positioned near the outer canthus of each eye (earth on forehead), connected to the computer A/D after suitable



amplification and filtering. On reaching a voltage threshold, the computer initiated the two-frame grating presentation at the beginning on the next frame. Observers indicated whether they saw the grating by pressing an appropriate button. The contrast of each presentation was near the estimated threshold (as determined by the Bayesian QUEST procedure²⁸), and final thresholds calculated by fitting Weibull functions²⁹ to the probability of seeing data. Standard errors, calculated by repeated estimates with partial data, were on average 0.05 log-units (less than symbol size). Note that the frequency cut-off is somewhat lower here than normal, probably owing to the brevity of the stimuli and the relatively lower luminance levels.



(confirming previous work⁶). For equiluminant stimuli, however, sensitivity during saccades was at least as high at all spatial frequencies as that in free viewing, and in some cases (notably with observer J.R.) even higher.

Figure 2 reports sensitivity for a forced-choice discrimination of the luminance or colour of a broad gaussian stripe, as the saccade progresses. Luminance discrimination (open circles) was strongly impaired at the beginning of the saccades, steadily recovering to normal levels about 150 ms later. But equiluminant stimuli (filled squares) showed higher sensitivity during saccades, reaching maximal levels at latencies of around 50–100 ms. This saccadic enhancement for chromatic stimuli was variable in magnitude but clearly evidenced by four observers (two not reported here) for both discrimination and detection (see also ref. 6).

We next sought to identify the level of visual analysis at which the suppression (of low-frequency luminance stimuli) occurs,

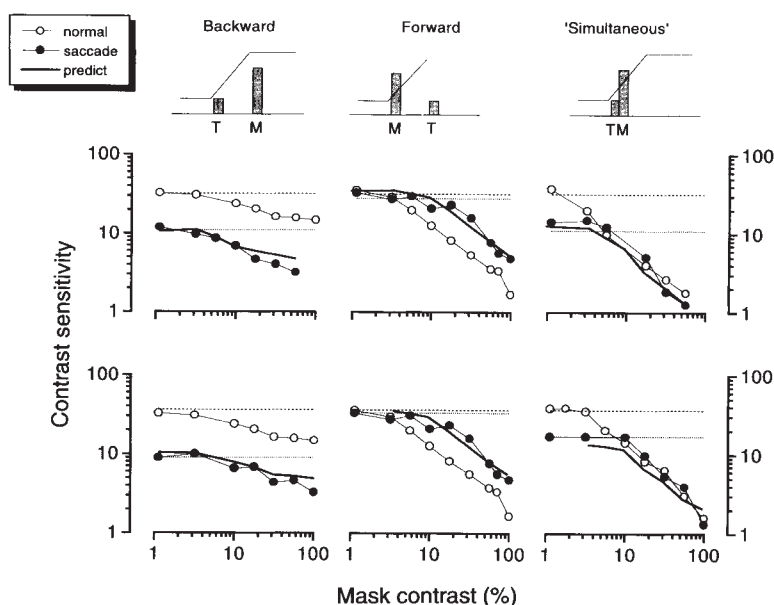
FIG. 2 Contrast sensitivity for discriminating whether a horizontal gaussian strip was brighter or darker (luminance condition: open circles) or redder or greener (colour condition: filled squares) than the background. The gaussian strip had space constant σ of 16.6° , and was presented for only one frame (8 ms). Data are reported as a function of latency after the beginning of a 40° saccade for two observers, one (J.F.) unaware of the goals of the experiment. Note that zero delay is not strictly the beginning of the saccade, but the beginning of the first frame of the monitor after the electro-oculogram had reached threshold voltage (~ 20 ms after the beginning of the saccade). Dashed lines indicate sensitivity for discrimination of luminance, and the dotted lines for colour during free viewing. Note that for both observers, luminance discrimination was severely impaired during saccades, steadily recovering to free viewing threshold, whereas the colour discrimination was enhanced during and for a period after the saccade. As before, the contrast of each presentation was placed near threshold by the QUEST procedure, and sensitivity determined by fitting the Weibull function (with over 200 trials). These measurements were made with the two-alternative forced-choice technique, so the 50% probability guessing factor was incorporated in the fit.

using a contrast masking paradigm, where either the test or the mask (or both) occurred during saccades (Fig. 3). With normal viewing, masks reduced sensitivity in all three masking conditions, as noted previously. Saccadic viewing changed the results in a quantitatively consistent way. With the backward masking condition, saccades reduced sensitivity at all contrasts by a factor of 3 (half a log-unit), suggesting that the test was suppressed before the site of contrast masking: suppression after masking would act on the 'masked' test and hence depend on mask contrast. With the forward-masking paradigm, the saccade-triggered sensitivities were systematically higher than those in free viewing, and well fitted by displacing the normal threshold curve by half a log-unit horizontally along the mask contrast axis. This also implies that saccadic suppression precedes contrast masking, as later suppression could not influence the masking process. The simultaneous masking results were also consistent with early suppression, as sensitivities during saccades were well fitted

FIG. 3 The effect of contrast masking on contrast sensitivity in saccadic and normal viewing conditions for two observers, D.C.B. (upper curves) and M.C.M. (lower curves). Observers were required to discriminate (two-alternative forced choice) the position of a briefly presented (8 ms) horizontally oriented grating patch centred 6.6° above or below the centre of the screen. The luminance $L(x, y)$ of the patch was given by

$$L(x, y) = L_0 + \exp(-x^2/2\sigma_x^2) \exp(-y^2/2\sigma_y^2) \cos(2\pi\omega y)$$

where L_0 is mean luminance, σ_x the horizontal space constant (16.7°), σ_y the vertical space constant (13.2°) and ω the carrier spatial frequency (0.075 c deg^{-1}). The mask, whose contrast is indicated on the abscissa, was a $67^\circ \times 53^\circ$ sinusoidal grating of the same spatial frequency and phase as the test, displayed for one frame (8 ms). The mask succeeded the test by 90 ms in the backward masking condition, preceded it by 90 ms in the forward masking condition, and followed immediately after in the 'simultaneous' condition. The open symbols represent measurements in free viewing, and the closed symbols measurements when the sequence was triggered by a 20° saccade. The dashed lines show thresholds with no mask in normal viewing, and the dotted lines during saccades. The thick lines show the predicted result if saccades attenuated contrast-gain before the site of masking: the normal viewing curves are displaced vertically by half a log-unit (attenuating test contrast) for backward masking, horizontally by the same amount (attenuating mask contrast) for forward masking and in both directions (attenuating both contrasts) for simultaneous masking. Again, the QUEST procedure positioned the contrast of each trial near threshold, and thresholds were calculated by fitting the



Weibull function to the 200-trial psychometric functions (incorporating the 50% guessing factor). The good agreement between the sensitivities during saccades and the predictions from scaling the normal viewing data strongly suggest that saccadic suppression precedes the site of contrast masking.

by displacing the normal results both vertically and horizontally by half a logarithmic unit, modelling the early attenuation of both test and mask. As much evidence suggests that contrast masking occurs mainly in primary visual cortex^{7,9}, saccadic suppression that precedes masking must occur at a very early visual site, making the lateral geniculate nucleus (LGN) a likely candidate.

The results of this study support earlier suggestions^{6,10,11} that motion mechanisms are selectively suppressed during saccades, thereby eliminating luminance transients of low spatial frequency that would otherwise become salient at saccadic speeds¹. In natural viewing conditions, this suppression could be supplemented by separate, visually driven mechanisms^{12,13} to attenuate further any residual visual signals generated by the saccade. Interestingly, effects of this type also seem to be confined to low-frequency patterns modulated in luminance^{14,15}.

There is now strong evidence for two functionally separate visual streams (magnocellular (M) and parvocellular (P)), originating in the retina, well preserved at the LGN, and continuing (with some crosstalk) through to associative cortex¹⁶. Although the degree of separability and the functions of the two pathways are still open to question, it is generally agreed that useful colour information is conveyed exclusively by the P-stream, and that the M-stream responds preferentially to transient and high-velocity stimuli of low spatial frequency^{17,21}. Our results (consistent with recent measurements of chromatic increment thresholds²²) suggest that saccades may selectively suppress the M-pathway (possibly at the level of the LGN) while sparing or even enhancing the P-pathway. As the M-pathway provides the major input to the putative motion centres²³ and to parietal associative areas²⁴ thought to be involved with visual atten-

tion^{25,26}, suppression of this pathway may blunt the disturbing sense of motion that saccades should otherwise elicit. □

Received 28 April; accepted 3 August 1994.

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ACKNOWLEDGEMENTS. This work was made possible by a special grant from the Department of Psychology at the University of Western Australia, and was supported by the Australian Research Council and the Italian Consiglio Nazionale delle Ricerche, target project "Robotica".

Calcium dependence of the rate of exocytosis in a synaptic terminal

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RAPID calcium-dependent exocytosis underlies neurotransmitter release from nerve terminals. Despite the fundamental importance of this process, neither the relationship between presynaptic intracellular calcium ion concentration ($[Ca^{2+}]_i$) and rate of exocytosis, nor the maximal rate of secretion is known quantitatively. To provide this information, we have used flash photolysis of caged Ca^{2+} to elevate $[Ca^{2+}]_i$ rapidly and uniformly in synaptic terminals, while measuring membrane capacitance as an index of exocytosis and monitoring $[Ca^{2+}]_i$ with a Ca^{2+} -indicator dye. When $[Ca^{2+}]_i$ was abruptly increased to $>10 \mu M$, capacitance rose at a rate that increased steeply with $[Ca^{2+}]_i$. The steepness suggested that at least four calcium ions must bind to activate synaptic vesicle fusion. Half-saturation was at $194 \mu M$, and the maximal rate constant was $2,000\text{--}3,000 s^{-1}$. A given synaptic vesicle can exocytose with high probability within a few hundred microseconds, if $[Ca^{2+}]_i$ rises above $100 \mu M$. These properties provide for the extremely rapid signalling required for neuronal communication.

Synaptic terminals of goldfish retinal bipolar neurons^{1,2} have been previously shown to be well-suited for the study of secretion using combined measurements of $[Ca^{2+}]_i$ and capacitance³. These techniques were used in single bipolar cell synaptic terminals filled with the photolysable Ca^{2+} -chelator DM-nitrophen⁴ through the whole-terminal patch pipette. A brief flash of light, given after spatial equilibration of DM-nitrophen, liberated Ca^{2+} from the chelator and increased $[Ca^{2+}]_i$. Figure 1 shows the response of a synaptic terminal to a light flash in such an experiment. The bottom trace shows on a slow timescale the change in $[Ca^{2+}]_i$ elicited by the flash, and the upper three traces show at high temporal resolution the resulting changes in capacitance, access conductance and membrane current. In this example, the flash caused a jump in $[Ca^{2+}]_i$ to about $40 \mu M$, as calculated from the fluorescence of the low-affinity Ca^{2+} indicator Fura-2⁵. After a 1.5-ms delay, capacitance increased rapidly after the flash, along an exponential time course with a time constant, τ , of 5.2 ms (corresponding to a rate constant, a , of $191 s^{-1}$). Neither access conductance nor membrane current changed.

The amount of Ca^{2+} liberated from DM-nitrophen, and thus the level of $[Ca^{2+}]_i$ achieved after a light flash, was graded with light intensity, as illustrated by the examples shown in Fig. 2. The dimmest flash (Fig. 2a) increased $[Ca^{2+}]_i$ to $\sim 18.5 \mu M$ but failed to elicit exocytosis. In the same terminal, a fivefold increase in flash intensity (Fig. 2c) produced a larger increase in $[Ca^{2+}]_i$ and a rapid increase in capacitance. Intermediate levels of $[Ca^{2+}]_i$ (Fig. 2b) evoked somewhat slower increases in capacitance. With unattenuated flashes (Fig. 2d), the calcium increase was greatest, and the capacitance rise was faster.

As an index of the rate of secretion, an exponential function was fitted to the rising phase of each capacitance response (for example, Fig. 1). In those terminals given multiple flashes, usu-

ally only the first response was evaluated in order to avoid complications of long-term Ca^{2+} effects. The resulting exponential rate constant (that is, the reciprocal of the time constant) is plotted against $[\text{Ca}^{2+}]_i$ for 43 experiments in Fig. 3a. Although there is considerable variability, the data can be well described by the cooperative binding of four calcium ions preceding a final,

Ca^{2+} -independent, secretory step, with the parameters given in the legend (Fig. 3a). Cooperative binding of three calcium ions leads to only marginal fit. Assuming independent binding, six or more calcium ions must be proposed; any fewer fail to match the steep rise and abrupt saturation. From the four- Ca^{2+} cooperative model, the calculated $[\text{Ca}^{2+}]_i$ at which the rate constant is

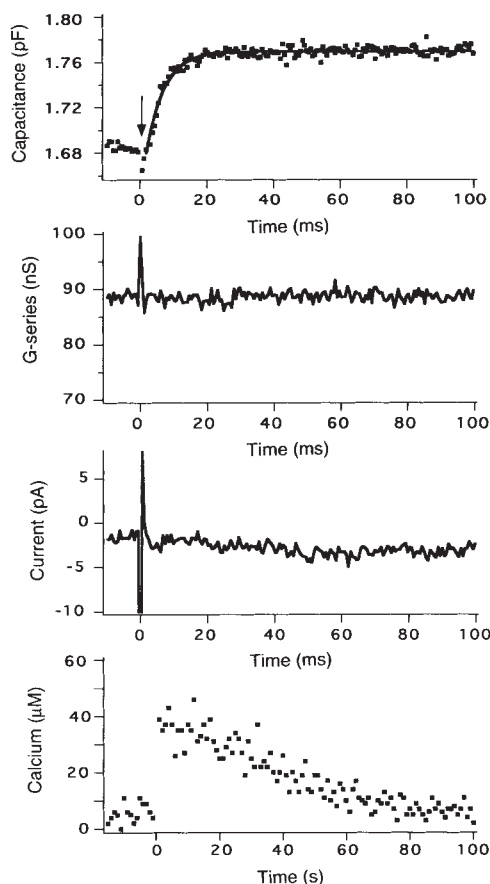
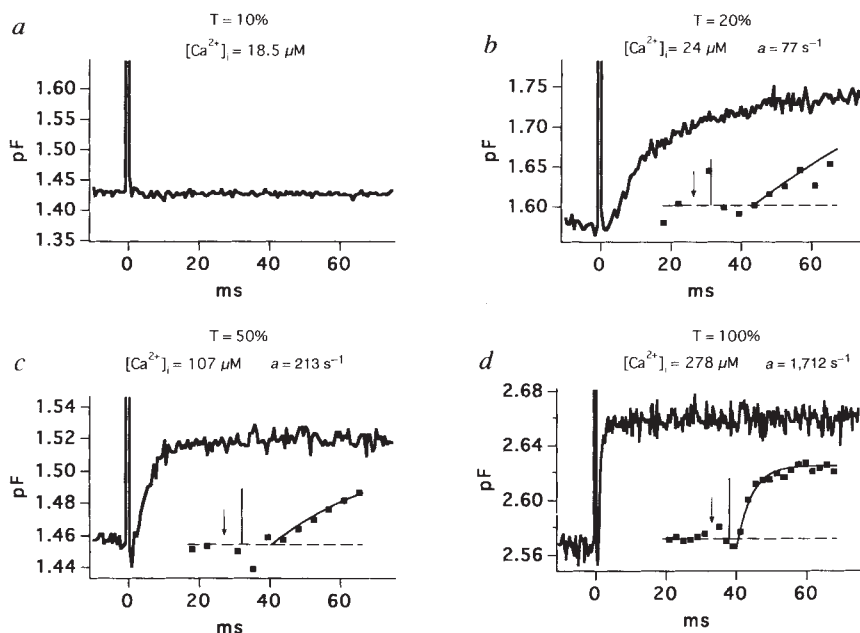


FIG. 1 Response of a bipolar cell synaptic terminal to a rapid increase of $[\text{Ca}^{2+}]_i$ after photolysis of Ca^{2+} -loaded DM-nitrophen. The resulting change in $[\text{Ca}^{2+}]_i$ is shown at low temporal resolution (bottom trace). Evoked changes in electrical properties, including membrane capacitance, are shown at high resolution (top traces). The UV flash was given at time = 0, indicated by the arrow (a) and by the flash artefact. The curve fitted to the capacitance response was drawn according to C (in pF) = $1.769 - 0.128(\exp(-at))$, where t is time after the flash and $a = 191 \text{ s}^{-1}$. In some terminals, a small Ca^{2+} -activated conductance increase was observed after the flash, but was not correlated temporally or in amplitude with the capacitance response. At still later times after the flash ($>1 \text{ s}$), slower components of increased capacitance were sometimes observed, coincident with an appreciable decline in $[\text{Ca}^{2+}]_i$ from its peak value. The average capacitance increase was $77 \pm 8 \text{ fF}$ (mean $\pm$ s.e.m., $n = 43$).

METHODS. Whole-terminal patch-clamp experiments were performed at $22\text{--}26^\circ\text{C}$ on synaptic terminals of goldfish retinal bipolar neurons within 1–2 h of dissociation^{3,22}. External bathing solution contained (mM): NaCl, 115; KCl, 2.6; MgCl_2 , 1.6; CaCl_2 , 1.0; glucose, 10; HEPES, 10; pH 7.3. Capacitance and series resistance were calculated²³ using automatic capacitance compensation of the EPC-9 amplifier²⁴ for slow measurements, and using a sinusoidal command voltage (1,600 or 3,200 Hz; 32 mV peak-to-peak; $V_{\text{hold}} = -60 \text{ mV}$) and a software emulation of a two-phase lock-in amplifier²⁵ for fast measurements. Internal solution contained (mM): DM-nitrophen, 10; CaCl_2 , 10; DTPA, 5; Magfura-2 (Fura-2), 1; Cs-glutamate, 62; Cs-HEPES, 31.2; TEA-Cl, 6.24; pH 7.27 ± 0.07 (mean $\pm$ s.d.). DM-nitrophen purity and Ca^{2+} saturation were experimentally determined²⁶. Preflash free $[\text{Ca}^{2+}]_i$ in internal solution was set to at least twice the resting $[\text{Ca}^{2+}]_i$ of synaptic terminals²² to inhibit endocytosis²⁷. Only the terminal and tip of the recording pipette were exposed to the UV light from the Xenon arc flash lamp (Rapp Optoelektronik, Germany). Photolysis of DM-nitrophen not loaded with Ca^{2+} produced no capacitance change ($n = 5$). Free $[\text{Ca}^{2+}]_i$ was measured as described^{14,28} with the fluorescent Ca^{2+} indicator Fura-2. We assume that with uniform illumination of spatially equilibrated DM-nitrophen, the globally measured $[\text{Ca}^{2+}]_i$ provides a reasonable estimate of $[\text{Ca}^{2+}]_i$ near the release sites.

FIG. 2 Changes in membrane capacitance in single synaptic terminals elicited by raising $[\text{Ca}^{2+}]_i$ with different flash intensities. The timing of the flash is indicated by the flash artefact at time = 0 or by arrows (insets). The numbers above each trace show the transmission (in %) of a quartz neutral density filter placed in the light path of the UV flash. $[\text{Ca}^{2+}]_i$ was calculated for a 400–800 ms interval, starting 100 ms after the flash. The rate constant, a , for each response was obtained by fitting a single exponential to the capacitance trace, as shown in Fig. 1. Data in a–c were obtained with a 1,600-Hz sine wave and a 190-V flash. Trace a and c are from the same terminal. Trace d was obtained with an unattenuated 225-V flash and a 3,200-Hz sine wave for better resolution of the rising phase. Insets (b–d) show 7 ms of the capacitance trace on an expanded time scale. Measurement of the delay is shown in the insets (b–d). Delays were measured as the time between the vertical line (see below) and the interception with the baseline (dashed line) of the single exponential fit of the capacitance rise (solid curve). Simulations of the effect of finite flash duration on the rate of capacitance rise demonstrated that the measured speed of exocytosis should not be grossly lowered by the calcium timecourse. The simulations did show, however, that the flash time course contributed $\sim 0.6 \text{ ms}$ to the delay of exocytosis. Placement of the vertical



line was corrected for this amount relative to the flash trigger (arrow), and an additional 0.1 ms, to allow time for Ca^{2+} release after photolysis²⁹.

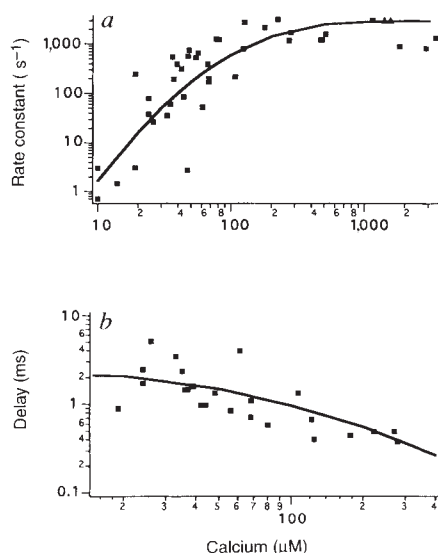


FIG. 3 *a*, Exponential rate constants for the rise of each capacitance response are plotted against post-flash $[Ca^{2+}]_i$. The rate constant, a , was obtained as in Fig. 1. In two terminals, the rising phase of the capacitance response was too fast to be fully resolved with a 3,200 Hz sine wave. These points are plotted as triangles at a fixed rate of 3,000 s^{-1} . The line is the predicted relationship based on an expansion of the kinetic model proposed for chromaffin cell secretion^{14,30}. Here, four sequential Ca^{2+} -binding steps are assumed, followed by a final secretion step. The initial pool of releasable vesicles was set to 77 fF (mean amplitude of the capacitance increase). The rate constant for the secretion step was set to 3,000 s^{-1} . A satisfactory fit (line) was obtained for a Ca^{2+} -binding rate constant of $14 \times 10^6 M^{-1} s^{-1}$ for all four steps, and a Ca^{2+} dissociation rate constant of 2,000 s^{-1} if only one calcium ion is bound. The dissociation rates for the second, third and fourth calcium ions were reduced with respect to the first by 0.4, 0.4² and 0.4³ respectively, introducing cooperativity into the model. Thus, the dissociation constant for the first calcium ion was 143 μM and for the last one 9.14 μM . Half-maximum rate in this model was reached at 194 μM . *b*, Delay vs $[Ca^{2+}]_i$. Delays were measured as in the legend of Fig. 2. The curve is the relationship predicted by the model, with parameters identical to those in *a*. Data shown are from representative terminals that responded to elevation of $[Ca^{2+}]_i$ with a capacitance response that had a readily resolvable rising phase. Four terminals with unusually long delays (30–40 ms) were excluded from the graph.

half-maximal is 194 μM . The $[Ca^{2+}]_i$ required for the activation of exocytosis was at least 10 μM , and typically greater than 20 μM , in agreement with previous results³. Maximal rates of secretion derived directly from the steepest part of the capacitance rises were 100,000–150,000 $fF s^{-1}$ at limiting $[Ca^{2+}]_i$. These correspond to the release of $\sim 1.2 \times 10^6$ vesicles s^{-1} , assuming a vesicle diameter of 50 nm (ref. 6). Such high rates are expected if a homogeneous pool of approximately 1,000 readily releasable vesicles (~ 17 vesicles per release site) is released with a mean latency of ~ 0.35 ms. Alternatively, a smaller pool of readily releasable vesicles might exist, to which neighbouring vesicles can fuse (compound exocytosis). The delay between the release of Ca^{2+} from DM-nitrophen and the start of the capacitance rise was measured for each terminal (for example, Fig. 2) and is plotted against $[Ca^{2+}]_i$ in Fig. 3*b*. The model parameters given in Fig. 3*a* for fitting the rate constants also faithfully predict the delays, suggesting Ca^{2+} dissociation rates between 2,000 s^{-1} and 128 s^{-1} , depending on the number of Ca^{2+} ions bound. Although faster rate constants, at a fixed ratio of on- and off-rates, also describe the steep relationship between the rate of secretion and $[Ca^{2+}]_i$, the predicted delays would then be very short compared with the actual delays.

Synaptic delays measured at intact synapses are in the sub-millisecond to millisecond time range^{7–9}. However, it is not clear whether this reflects the high intrinsic speed of the process of exocytosis¹⁰, the fast rise and fall of a driving force (such as $[Ca^{2+}]_i$), or the combination of rapid changes in $[Ca^{2+}]_i$ and voltage¹¹. The data presented here document two features, which according to basic considerations of reaction kinetics, should permit fast turn-on and fast decay of neurotransmitter release: high maximal rates and low Ca^{2+} affinity (implying fast dissociation rates). Bipolar cell synaptic terminals form ribbon-type synapses⁶, which allow for tonic, but rapidly changing signal transfer in the retina. Although they represent a specialized type of synapse, they are the only synaptic terminals on which measurements of this kind can be made. Similar quantitative data have been obtained in neuroendocrine cells from the pituitary^{12,13} and adrenal glands¹⁴. Compared with these, synaptic terminals show higher maximum rates, enabling terminals to secrete faster, and require higher calcium, consistent with a lower-affinity Ca^{2+} trigger for exocytosis. The maximum secretion rates are about 3,000 s^{-1} in synaptic terminals, whereas in adrenal chromaffin cells they were $\sim 1,000 s^{-1}$. In pituitary cells, they were 20–50 s^{-1} . The minimum $[Ca^{2+}]_i$ at which secretion can be observed is around 0.5–1 μM for chromaffin cells and pituitary cells^{12,14}, whereas it is 10–20 μM in synaptic terminals. $[Ca^{2+}]_i$ at active zones is believed to reach several hundred micromolar during exocytosis^{15–17}, sufficient for obtaining maximal release rates. As shown by Ca^{2+} diffusion models, this is achieved through a distinct co-localization of channels and release sites^{18–20}. Such microdomains build up and collapse within microseconds after opening or closing of Ca^{2+} channels. Owing to the low affinity of the Ca^{2+} trigger site, the Ca^{2+} binding state would be able to follow these fast Ca^{2+} changes on the microsecond to millisecond timescale. The properties of the release sites described here, together with the concept of Ca^{2+} microdomains^{15–21}, therefore fulfil all the requirements for fast neuronal signalling. □

Received 23 May; accepted 15 August 1994.

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ACKNOWLEDGEMENTS. This work was supported by a fellowship of the Alexander von Humboldt Foundation to R.H., a fellowship of the Boehringer Ingelheim Foundation to C.H., and by NIH grants to G.M. We thank J. Herrington and R. J. Bookman for the use of their Pulse Control extensions to IGOR. R. Chow for the use of his capacitance-calculation macros for IGOR, and M. Naraghi for advice on model calculations.

A new class of ligand-gated ion channel defined by P_{2x} receptor for extracellular ATP

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EXTRACELLULAR ATP exerts its effects through P_2 purinoceptors¹: these are ligand-gated ion channels (P_{2x})^{2,3} or G-protein-coupled receptors (P_{2y} , P_{2u})⁴. ATP at P_{2x} receptors mediates synaptic transmission between neurons⁵⁻⁷ and from neurons to smooth muscle¹, being responsible, for example, for sympathetic vasoconstriction in small arteries and arterioles^{8,9}. We have now cloned a complementary DNA encoding the P_{2x} receptor from rat vas deferens and expressed it in *Xenopus* oocytes and mammalian cells. ATP activates a cation-selective ion channel with relatively high calcium permeability. Structural predictions suggest that the protein (399 amino acids long) is mostly extracellular and contains only two transmembrane domains plus a pore-forming motif which resembles that of potassium channels. The P_{2x} receptor thus defines a new family of ligand-gated ion channels.

ATP evoked inward currents in oocytes injected with poly(A)⁺ RNA from rat vas deferens (Fig. 1a) and urinary bladder, but not in uninjected oocytes. We generated a unidirectional cDNA library from the poly(A)⁺ RNA of vas deferens, and screened oocytes injected with RNA transcribed *in vitro* from progressive subdivisions of this library. A single clone was isolated that gave

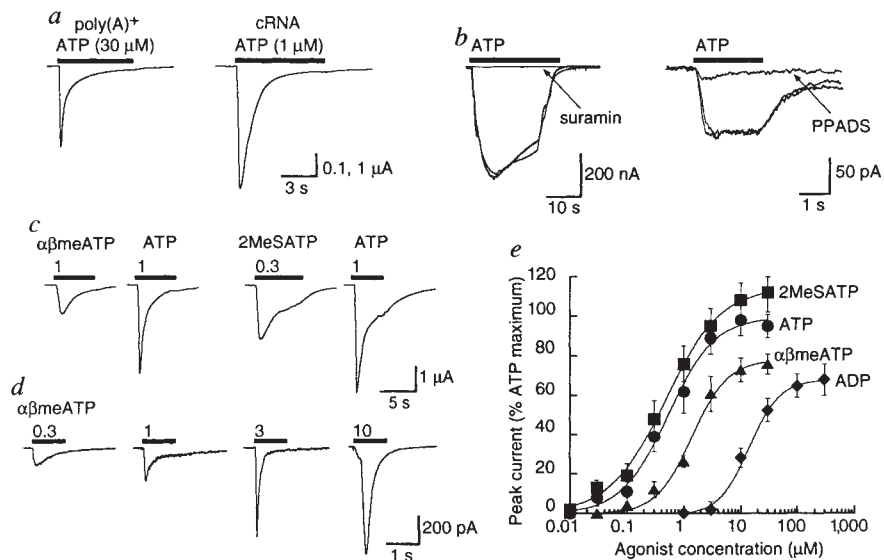
ATP-evoked currents; it carried an insert of 1,837 base pairs (bp). The cDNA was also transiently expressed in human embryonic kidney (HEK293) cells.

ATP, $\alpha\beta$ -methylene-ATP, 2-methylthio-ATP and ADP evoked inward currents with short latency (minimum <2 ms) and fast rise time (10–90%, 7 ± 0.2 ms at 10 μ M ATP in five HEK293 cells). Currents were sustained with low agonist concentrations (10–30 nM), but declined during the application of higher concentrations (Fig. 1c, d). As for native smooth-muscle cells^{3,10}, repeated applications of ATP (≥ 1 μ M) evoked progressively smaller currents unless separated by at least 8 to 12 min. The order of agonist potency was 2-methylthio-ATP $\geq$ ATP $>$ $\alpha\beta$ -methylene-ATP $>$ ADP (Fig. 1e). UTP (100 μ M, $n=3$), GTP (100 μ M, $n=3$), acetylcholine (100 μ M, $n=3$) and 5-hydroxytryptamine (50 μ M, $n=3$) were ineffective. Currents evoked by ATP, $\alpha\beta$ -methylene-ATP, 2-methylthio-ATP and ADP were reversibly blocked by suramin (3–100 μ M) and by pyridoxalphosphate-6-axophenyl-2',4'-disulphonic acid (PPADS, 10–30 μ M, $n=7$; Fig. 1b)¹¹, but not by amiloride (100 μ M, $n=4$). These pharmacological properties define the expressed receptor as a P_{2x} purinoceptor^{1,12}.

The current evoked by ATP reversed polarity at zero voltage in control solution (0.2 ± 5.2 mV, $n=4$ oocytes; -3 ± 0.8 mV, $n=4$ HEK293 cells); this reversal potential (E_{rev}) shifted by ~ 12 mV when the external Na^+ concentration was halved (-12.1 ± 3.4 mV, $n=5$ oocytes; -15 ± 2 mV, $n=4$ HEK293 cells; Fig. 2a). E_{rev} changed from -33.9 ± 1.3 mV ($n=5$ oocytes) to -22.3 ± 1.4 mV ($n=4$ oocytes) when the K^+ concentration was changed from 2 to 20 mM (in 20 mM sodium), but did not change when external chloride was replaced with methane sulphonate, gluconate or isethionate ($n=8$ oocytes). With 150 mM internal and external Na^+ , E_{rev} was -0.8 ± 0.3 mV ($n=4$ HEK293 cells); this shifted to 14.5 ± 1.2 mV when external Na^+ was replaced with Ca^{2+} (Fig. 2b). From these values, the ratio of Ca^{2+} to Na^+ permeability was 4.8 ± 0.4 (Fig. 2). ATP

FIG. 1 a, Inward currents evoked by ATP in oocytes injected with poly(A)⁺ RNA from rat vas deferens (left; 0.1 μ A calibration) and RNA transcribed from the P_{2x} cDNA clone (right; 1 μ A calibration). b, Currents recorded in oocytes (left, 50 nM) and HEK293 cells (right, 100 nM) in response to ATP before, during and after wash-out of suramin (10 μ M) and PPADS (30 μ M). c, Responses to 2-methylthio-ATP (2MeSATP; 0.3 μ M) and $\alpha\beta$ -methylene-ATP ($\alpha\beta$ meATP; 1 μ M) compared with those to ATP (1 μ M) in the same oocytes. d, Currents evoked by $\alpha\beta$ meATP (μ M) in one HEK293 cell. e, Concentration–response relations for agonists in HEK293 cells. Currents are normalized to that caused by 30 μ M ATP in the same cell; each point is mean $\pm$ s.e. of 3–8 cells.

METHODS. Total RNA was isolated by the guanidinium isothiocyanate method from vas deferens of 4-week-old Sprague-Dawley rats, and poly(A)⁺ RNA was purified by oligo(dT) cellulose. First-strand cDNA primed with 5'-(GA)₅GCGGCCG(T)₁₅-3' was synthesized with Superscript and converted to double-stranded DNA. EcoRI linkers were ligated to the cDNA and the product was digested with NotI. The EcoRI–NotI cDNA of 1.3–9 kb was isolated by gel electrophoresis, and a unidirectional library was constructed by ligation of the cDNA to pBCKMV. The library was electroporated into *E. coli* DH10B cells and divided into 24 pools of 8×10^4 clones. Plasmid DNA from the pools was prepared by mini-alkaline lysis followed by LiCl precipitation. NotI-linearized cDNA was transcribed *in vitro* with T3 RNA polymerase in the presence of cap analogue m⁷G(5')ppp(5')G. The *in vitro* transcribed RNA (50 nl of 4 mg ml⁻¹) was injected into defolliculated *Xenopus* oocytes and two-electrode voltage-clamp recordings were made after 2–6 days. For screening cDNA pools, the solution contained (in mM): NaCl 96, KCl 2,



CaCl₂ 1.8, MgCl₂ 1, sodium pyruvate 5, HEPES 5, pH 7.6. Characterization of the cloned P_{2x} receptor was performed on oocytes injected with 75 ng cRNA, with Ba²⁺ replacing Ca²⁺ to inhibit endogenous calcium-activated chloride currents. Microelectrodes contained 3 M KCl (0.5–2 M Ω). HEK293 cells were transfected by lipofection²⁵; ATP (30 μ M) had no effect on 45 non-transfected cells. Whole-cell currents were recorded at room temperature; patch pipettes (5 M Ω) contained (mM) Cs- or K-aspartate 140, NaCl 5, EGTA 11, HEPES 5. External solution was NaCl 150, KCl 2, CaCl₂ 2, MgCl₂ 1, HEPES 5, glucose 11; pH and osmolality were maintained at 7.3 and 305 mosmol l⁻¹ respectively. Complete solution exchange could be achieved within 1 ms.

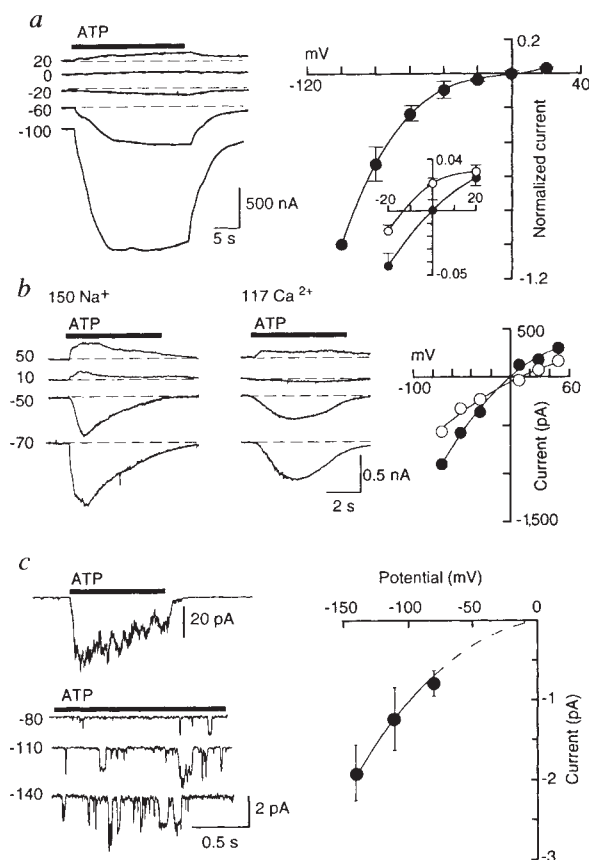


FIG. 2 *a*, ATP-evoked currents in one oocyte at indicated holding potentials (mV); ATP (30 nM) was applied at 8-min intervals. Current-voltage (*I/V*) relation shows 4 similar experiments; currents normalized to that at -100 mV. Inset: *I/V* expanded around 0 mV for control (filled circles) and low- Na^+ solutions (48 mM, *N*-methyl-D-glucosamine substitution). Points are mean $\pm$ s.d. for 4–5 experiments. *b*, ATP-evoked currents in one HEK293 cell under bi-ionic conditions at the holding potentials indicated (mV); ATP (100 nM) was applied at 12-min intervals. *I/V* relation shows currents recorded in sodium (filled circles) or calcium (open circles); reversal potential is about 12 mV positive in calcium to that in sodium. *c*, Currents evoked by ATP in outside-out membrane patches from injected oocytes. Upper trace, an initial application of ATP (30 nM, 1 s, -140 mV) opens many channels. Lower traces, typical responses to subsequent ATP applications, at holding potentials indicated (mV). Graph plots single-channel *I/V* relation obtained from resolvable unitary currents recorded from 73 patches in 14 oocytes. Points are means and s.d. of gaussians fitted to amplitude distributions at the three potentials. METHODS. Solutions for experiments examining Ca^{2+} permeability of ATP currents in HEK293 cells contained (mM): internal solution, NaCl 150, HEPES 5, CaCl_2 0.5, EGTA 5 (free Ca^{2+} concentration about 5 nM); external Na^+ solution, NaCl 150, glucose 11, histidine 5, CaCl_2 2; external Ca^{2+} solution, CaCl_2 117, glucose 11, histidine 5. pH and osmolarity of solutions were 7.4 and 295 mosmol l^{-1} respectively. $P_{\text{Ca}}/P_{\text{Na}}$ was computed from $a_{\text{Na}}[\text{Na}]_i \{ \exp(E_{\text{rev}}F/RT)(1 + \exp(E_{\text{rev}}F/RT)) / 4a_{\text{Ca}}[\text{Ca}]_o (RT/F = 25.2 \text{ mV})^{10,26} \}$; E_{rev} was corrected for liquid junction potential; concentrations were converted to activities using $a_{\text{Na}} = 0.75$ and $a_{\text{Ca}} = 0.25$ (ref. 10). For single-channel recordings, pipettes (5–10 M Ω) contained (mM) K-gluconate 115, HEPES 5, BAPTA 5 and MgCl_2 0.5; external solution as for Fig. 1. ATP was applied typically for 1 s; data was sampled at 5 kHz (filtered at 1 kHz) in 2-s segments beginning 300 ms before onset of agonist application.

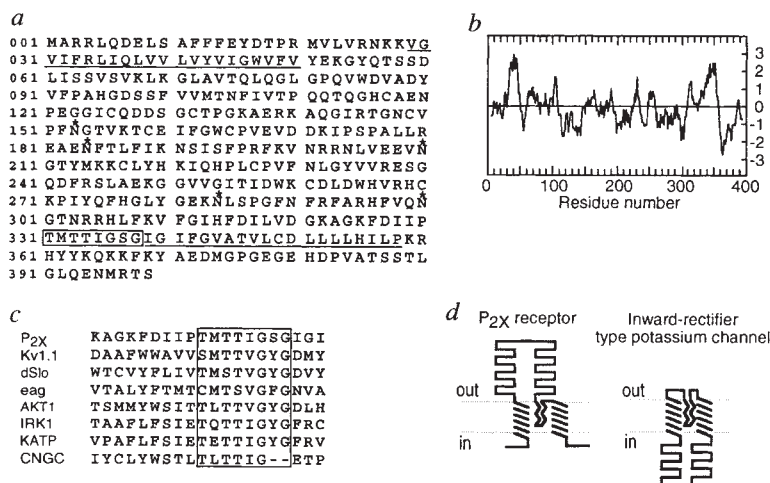
(30 nM) evoked unitary currents in 44 of 73 (64%) outside-out membrane patches (Fig. 2c); the effect of ATP declined markedly with subsequent applications to the same patch. The chord conductance was 19 pS between -140 and -80 mV (Fig. 2c). Channel activity was not seen in suramin (30 μM) or in non-injected oocytes. These electrophysiological properties of the cloned $\text{P}_{2\text{X}}$ receptor closely resemble those of smooth muscle^{2,3,10}, suggesting that a single subunit forms fully functional channels.

The nucleotide sequence of the $\text{P}_{2\text{X}}$ receptor cDNA had a single open reading frame encoding 399 amino acids (Fig. 3a).

FIG. 3 *a*, Predicted amino-acid sequence of the $\text{P}_{2\text{X}}$ receptor. Underlined sequences indicate hydrophobic regions; the putative pore is boxed. Potential sites of *N*-glycosylation are marked with asterisks. *b*, Hydrophobicity plot of the predicted amino-acid sequence of the $\text{P}_{2\text{X}}$ receptor. Hydrophobicity values were calculated according to Kyte and Doolittle and are plotted against amino-acid position (window size, 9 amino acids); positive values indicate hydrophobic regions. *c*, Alignment of the proposed pore region of the $\text{P}_{2\text{X}}$ receptor with that of a rat voltage-dependent K^+ channel (Kv1.1), a *Drosophila* Ca^{2+} -activated K^+ channel (dSlo), a *Drosophila* cyclic-nucleotide-gated K^+ / Ca^{2+} channel (eag), an *Arabidopsis* K^+ channel (AKT1), a mouse inward-rectifier K^+ channel (IRK1)²⁷, a rat K^+ channel regulated by intracellular ATP (K-ATP)¹⁶, and a rat cyclic-nucleotide-gated cation channel (CNGC)^{15,17}. *d*, Schematic representation of the membrane topology for K^+ channels of the inward-rectifier and G-protein-activated types^{16,17,27,28}.

METHODS. cDNA was sequenced by the exonuclease III procedure²⁹. Data obtained by fluorescent DNA sequencing (Applied Biosystems) were compiled with Sequencher 2.1 (Gene Codes Corporation, MI). The nucleotide sequence has been deposited in EMBL, accession number X80477.

The ATG occurred in a consensus for translation initiation, and charged residues in the first 28 amino acids suggest the absence of a secretion leader. The computed molecular weight of the polypeptide (M_r , 45K) agrees with that of the *in vitro* translation product labelled with ³⁵S-methionine (data not shown). The higher molecular weight of the $\text{P}_{2\text{X}}$ receptor solubilized from rat vas deferens (62K)¹³ may reflect glycosylation. Two hydrophobic regions are sufficiently long to cross the membrane (Fig. 3b); the 287-amino-acid hydrophilic region connecting these two putative membrane-spanning regions has five potential *N*-linked glycosylation sites and ten cysteine residues and contains a



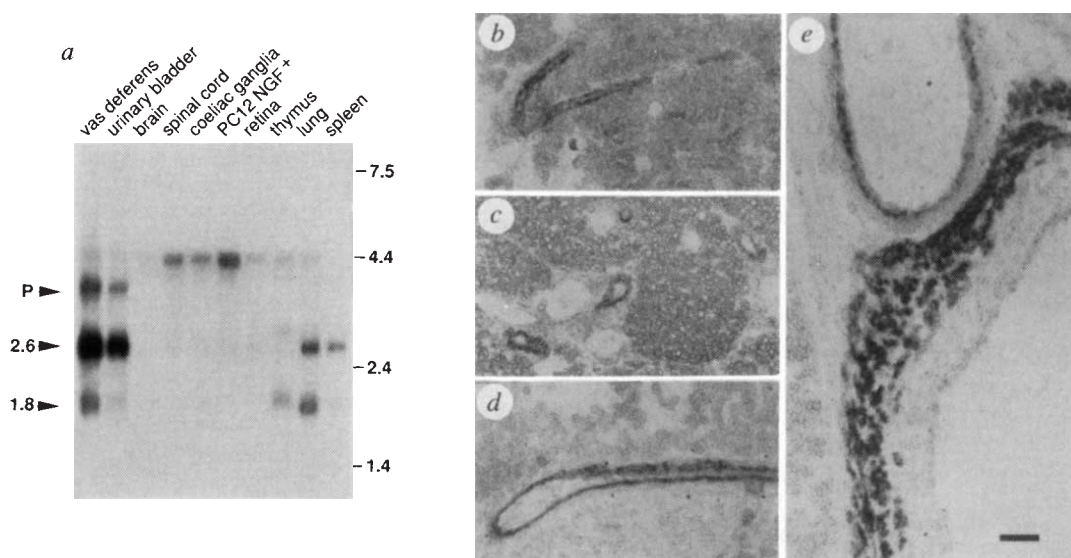


FIG. 4 a, Tissue distribution of the P_{2X} receptor mRNA in rat. Lanes contained either 50 ng poly(A)⁺ RNA (vas deferens and urinary bladder) or 1 µg poly(A)⁺ RNA (all others). The apparent shift in migration for mRNA species in thymus was observed in three independent experiments. b–e, Cellular localization of the P_{2X} receptor mRNA by *in situ* hybridization. Panels show digoxigenin-riboprobe hybridization in the smooth muscle layer of a lingual arteriole (b), a trapezius muscle artery (c), the carotid artery (d), and the smooth muscle layer of the urinary bladder and umbilical artery (e). METHODS. After electrophoresis in 1% agarose/6% formaldehyde, RNA samples were transferred to nylon membranes and quantified by methylene blue staining. This northern blot was hybridized with a ³²P-labelled riboprobe corresponding to the entire sequence shown in Fig. 3, washed in 0.2 × SSC + 1% SDS at 60 °C and incubated with RNase

A to remove nonspecifically bound material. The sizes of M_r markers (right) are from an RNA ladder (Bethesda Research). The sizes of mRNA species (left) are for the corresponding cDNAs from the vas deferens. Sagittal cryosections (16 µm) from 2-day-old rats (b–d) or E19 embryos (e) were hybridized at high stringency (50% formamide + 5 × SSC, 72 °C) with 50 ng ml⁻¹ digoxigenin-UTP-labelled cRNA probe for the P_{2X} receptor³⁰. Both sense and antisense riboprobes were tested and no sense strand hybridization was seen. Hybridization was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase using NBT/BCIP substrate solution (75 mg ml⁻¹ 4-nitroblue tetrazolium chloride and 50 mg ml⁻¹ 5-bromo-4-chloro-3-indolylphosphate; Boehringer Mannheim). Results are shown for 18-h phosphatase reactions. Scale bar, 100 µm.

sequence (residues 131–144) similar to the Walker type-A ATP-binding motif [G(X₄)GK(X₇)(I/V)]¹⁴. This suggests that the N and C termini are intracellular, with most of the protein in the extracellular domain.

An 8-amino-acid sequence of the P_{2X} receptor in the region preceding the second putative transmembrane domain contains a sequence (TMTTIGSG) that closely resembles a motif which is conserved among all voltage-gated potassium channels, inward rectifier potassium channels, and cyclic-nucleotide-gated cation channels^{15,16} (Fig. 3c). In potassium channels, this short sequence (P or H5 segment) forms the inner lining of the extracellular half of the ionic pore¹⁷. We propose that this P-like segment forms the narrowest part of the P_{2X} receptor channel pore (Fig. 3d).

RNA blot analysis demonstrated hybridization of four different bands with estimated messenger RNA sizes of 1.8, 2.6, 3.6 and 4.2 kilobases (kb) (Fig. 4a); the strongest signals were in vas deferens and urinary bladder. The smallest mRNA (1.8 kb) corresponded in size to our cloned P_{2X} receptor. The predominant mRNA in bladder and vas deferens (2.6 kb) has a 3' untranslated extension, as determined by sequencing other cDNA clones (not shown). Both the 2.6-kb and 1.8-kb mRNAs were found in lung and spleen; this hybridization could result from smooth muscle in these organs. The 2.6-kb transcript gave a faint signal in spinal cord, coeliac ganglia and nerve growth factor(NGF)-differentiated PC12 cells. The 3.6-kb mRNA apparent in the vas deferens and urinary bladder (indicated by P in Fig. 4a) may represent a precursor for P_{2X} mRNA, as it was observed with a P_{2X} intron-specific probe (not shown). *In situ* hybridization with antisense P_{2X} cRNA showed staining of the urinary bladder, and the smooth muscle layers of small arteries and arterioles (Fig. 4b–e).

Ion channels gated by extracellular ligands (nicotinic, serotonin 5-HT₃, GABA_A, glycine, and excitatory amino-acid receptors) share the same overall topology ('three-plus-one' transmembrane spans and a long extracellular N terminus), and within subfamilies there is considerable sequence conservation^{15,18}. The P_{2X} receptor is quite unlike those channels; it has only two hydrophobic segments sufficiently long to cross the membrane and these are separated by a large, cysteine-rich, presumably extracellular domain. Such a molecular architecture has been proposed for the mechanosensitive channels of *Caenorhabditis elegans*¹⁹ and the related rat amiloride-sensitive epithelial sodium channel²⁰. There is, however, no primary sequence homology between those channels and the P_{2X} receptor.

The P_{2X} receptor cDNA sequence suggests a surprising connection with apoptosis. A partial cDNA clone (RP-2) was previously isolated by subtractive hybridization from thymocytes; RP-2 RNA was selectively expressed by cells induced to die²¹. RP-2, from base 38 to its end, is identical to the P_{2X} receptor cDNA; the first 37 nucleotides of RP-2 are within an intron that we sequenced from a vas deferens cDNA, indicating that RP-2 is a fragment of an incompletely processed P_{2X} receptor gene. Could extracellular ATP initiate programmed cell death through its action at P_{2X} receptors? ATP induces cell death in thymocytes²², hepatocytes²³ and several lymphocytic cell lines²⁴, apparently by increasing the intracellular calcium concentration. In those cases P_{2Z} or P_{2Y} purinoceptors appear to be involved, but apoptosis may also result from calcium entry through P_{2X} receptors in much the same way as occurs in neuronal cell death following glutamate action at N-methyl-D-aspartate receptors. The results imply that the P_{2X} receptor and related molecules may have functional roles not only in synaptic transmission but also in programmed cell death. □

Received 6 June; accepted 3 August 1994.

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ACKNOWLEDGEMENTS. We thank E. Andre and M. Abdallah for *in situ* analysis, M. Solazzo and F. Talbot for sequencing, L. Potier for dissections, D. Estoppey for cell culture, G. Lamprecht for PPADS, and H. Robert Guy for discussion.

New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor

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THE adenosine-5'-triphosphate (ATP) molecule is an extracellular messenger in neural and non-neural tissues, where it activates several cell-surface-receptor subtypes, including G-protein-coupled receptors and ligand-gated ion channels¹. ATP-gated channels (termed P_{2X} receptors) have been characterized on smooth muscle cells and autonomic and sensory neurons, where they mediate membrane depolarization and, in some cases, Ca²⁺ entry². P_{2X} receptors are functionally heterogeneous, but resemble acetylcholine- and serotonin-gated channels with respect to ion selectivity and kinetic parameters of channel gating. We report here that despite such close functional similarities, the deduced sequence of a cloned P_{2X} receptor predicts an unusual subunit structure resembling voltage-insensitive cation channels. Thus, the P_{2X} receptor provides a striking example of convergent evolution, whereby proteins have been fashioned with similar functional properties from subunits having very different structural characteristics. There is sequence similarity between the ATP receptor and *RP-2*, a gene activated in thymocytes undergoing programmed cell death³. *RP-2* may encode a receptor for ATP or another metabolite released during apoptosis.

Fast excitatory responses to ATP have been extensively characterized in rat pheochromocytoma PC12 cells^{4–7}. A complementary DNA expression library constructed from PC12 messenger RNA was screened for the production of ATP-evoked inward current responses in oocytes, and a single cDNA clone was isolated. Currents produced in oocytes injected with cRNA transcripts derived from this clone are shown in Fig. 1a. Prolonged application of ATP elicited a large inward current with a rapid onset and slow rate of desensitization. The existence of multiple P_{2X} subtypes has been inferred from the differential actions of agonists such as α,β -methylene-ATP and 2-methylthio-ATP². The pharmacological profile of the cloned receptor was determined using these compounds, as well as a variety of other nucleoside and nucleotide analogues (Fig. 1a). ATP, ATP- γ S and 2-methylthio-ATP (2-MeS-ATP) were found to be roughly equipotent as agonists, whereas α,β -methylene-ATP and β,γ -methylene-ATP were inactive as agonists or antagonists.

By these criteria, the cloned receptor resembles native P_{2X} receptors on PC12 cells and some sensory and autonomic neurons^{4,6,8,9}, but differs from those on vascular smooth muscle, vas deferens, and some central nervous system (CNS) neurons, where α,β -methylene-ATP acts as a potent agonist^{10–14}. Among the many nucleotide and nucleoside derivatives examined, only CTP and dATP elicited small but detectable currents, whereas ADP, AMP, 5'-adenylylamido-diphosphate, adenosine, GTP, UTP, cyclic AMP and cyclic GMP were inactive. The neurotransmitters acetylcholine, glutamate, glycine, γ -aminobutyric acid (GABA) and serotonin failed to activate the cloned receptor. The non-subtype selective P₂ receptor antagonists suramin and reactive blue-2 have been used to distinguish P_{2X} receptors from other ligand-gated ion channels¹⁵. Both of these compounds reversibly antagonized ATP-evoked responses by over 95% (Fig. 1b). d-Tubocurarine, a potent antagonist of acetylcholine- and serotonin-gated channels, only partially blocked (~50%) ATP-evoked responses.

Dose-response analysis of the cloned receptor (Fig. 1c) revealed a half-maximal effective concentration (EC₅₀) for ATP of 60 μ M and a Hill coefficient of 2.0, suggesting that activation of the cloned receptor-ion-channel complex requires the binding of more than one agonist molecule. Extracellular Zn²⁺ has been shown to potentiate ATP-activated currents in rat sensory and sympathetic neurons, possibly by increasing the affinity of P_{2X} receptors for agonist^{9,16}. Addition of 10 μ M Zn²⁺ to the bathing solution had a similar effect on the cloned receptor, shifting the EC₅₀ for ATP to 15 μ M.

The current-voltage relationship of the cloned P_{2X} receptor (Fig. 1d) shows a marked inward rectification with a reversal potential of about –5 mV. Replacement of all extracellular monovalent cations by the large organic cation Tris, shifted the reversal potential to –45 mV and reduced the current amplitude significantly. Replacement of all extracellular Na⁺ by K⁺ did not affect the reversal potential. These results suggest that, like native P_{2X} receptors^{7,8}, the cloned receptor incorporates an inwardly rectifying, nonspecific cation channel equally permeable to Na⁺ and K⁺, allowing conduction of even large cations. Finally, replacement of all extracellular Ca²⁺ by Ba²⁺ had no effect on the observed current, indicating that there is no significant contribution from endogenous Ca²⁺-activated Cl[–] channels.

pP_{2X}R1 contains a 1,416-nucleotide open reading frame encoding a 472-amino-acid protein with a predicted M_r of 52,557 (Fig. 2a). The deduced amino-acid sequence bears no homology with any known receptor protein, but certain key structural features and overall transmembrane topology of P_{2X}R1 bear striking resemblance to a growing family of protein subunits that form inwardly rectifying and pH-sensitive K⁺ channels, amiloride-sensitive Na⁺ channels, and presumptive mechanosensitive ion channels from *Caenorhabditis elegans*^{17–22}. Thus, P_{2X}R1 contains two hydrophobic putative transmembrane

domains (M1 and M2) with an intervening hydrophilic loop of ~270 residues. The apparent absence of an amino-terminal signal sequence suggests a model (Fig. 2b) in which both the N- and C-termini are in the cytoplasm and the hydrophilic cysteine-rich loop resides outside the cell. These regularly spaced cysteine residues could form disulphide-bonded hairpin structures which might be important for stabilizing a ligand-binding pocket, reminiscent of the so-called 'Cys-Cys loop' motif found in nicotinic acetylcholine receptors and other members of the ligand-gated ion channel superfamily²³. Pertinent to this topological assignment is the fact that all three potential N-linked glycosylation sites are located in the hydrophilic loop region. Presumably, ATP binds to a site(s) within the extracellular loop. A search of this domain for potential nucleotide-binding sites revealed two segments that resemble a Walker type-A phosphate-binding motif²⁴. Finally, the proline- and serine-rich C-terminal tail con-

tains sites at which channel activity could be modulated by phosphorylation.

Biochemical and genetic data suggest that within this family of cation channels the M2 domain, together with an adjacent hydrophobic segment (H5), forms the ion pore and ion-binding sites (reviewed in ref. 25). In some cases, the H5 segment has been proposed to assume a β -sheet structure that is preceded by a turn²². A similar configuration can be seen within the proposed P_{2X}R1 structure. Genetic studies of *C. elegans* touch mutants suggest that M2 forms an amphipathic α -helix whose polar residues project into the ion pore^{19,20}. Although P_{2X}R1 M2 shares no obvious sequence similarity with other cation channels, a helical wheel plot illustrates the potential of this domain to form an amphipathic α -helix (Fig. 3b). By analogy with the amiloride-sensitive Na⁺ channel, which consists of at least three distinct subunits¹⁷, ATP-gated ion channels are also

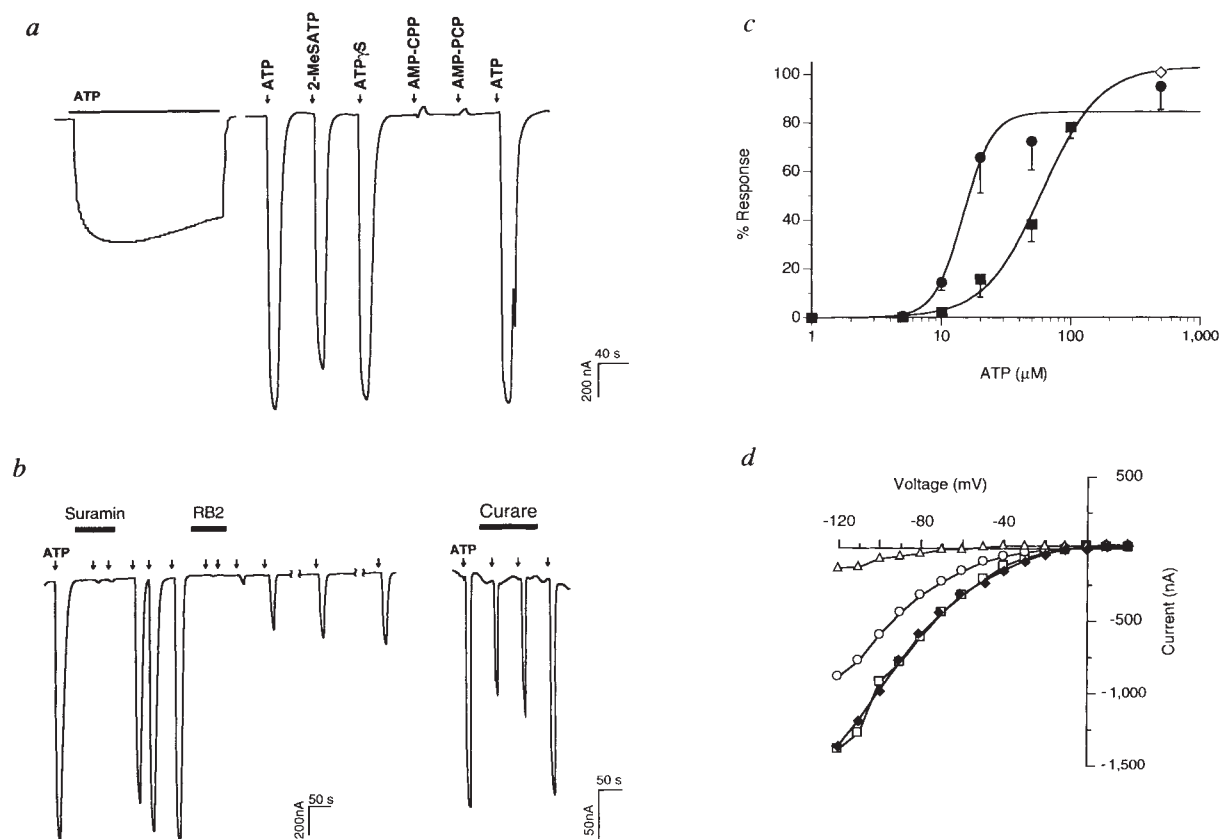


FIG. 1 Pharmacological and electrophysiological properties of the P_{2X}R1 receptor expressed in *Xenopus* oocytes. **a**, Currents recorded in *Xenopus* oocytes injected with P_{2X}R1 cRNA (~10 pg) in response to ATP and other P_{2X} agonists. Oocytes were voltage-clamped at -70 mV. Agonists (50 μ M) were bath-applied for the period indicated (bar) or in 10-s pulses (arrow). **b**, Effect of P_{2X} antagonists on ATP-evoked membrane currents. ATP (50 μ M) was applied in 10-s pulses (arrow), in the absence or presence (solid bar) of the antagonists suramin (100 μ M), reactive blue-2 (RB2; 100 μ M), or curare (1 μ M). Recovery of ATP-evoked responses following a wash-out period of each antagonist is shown. For RB2, the total wash-out period was 15 min. **c**, Dose-response curve for ATP in the absence and presence of extracellular Zn²⁺. Oocytes were voltage-clamped at -80 mV and ATP applied at various concentrations in 10-s pulses. Cells were allowed a recovery period of 2 min between agonist applications. Responses were measured in the absence (squares) and presence (circles) of 10 μ M ZnCl₂ in the bathing solution. Values represent the mean of peak current responses of three independent cells, each normalized to the response evoked by 500 μ M ATP in the absence of Zn²⁺ (open diamond). Curves were fitted by nonlinear regression analysis (Enzfitter) to the Hill equation, resulting in EC₅₀ values of 59.9 ($\pm$ 5.3) and 14.8 ($\pm$ 1.9) μ M in the absence and presence of Zn²⁺, respectively. Hill coefficients were 2.0

($\pm$ 0.3) and 3.8 ($\pm$ 1.4), respectively. **d**, Current-voltage relationship. Currents were measured in response to ATP (50 μ M; 10-s pulse) in cells voltage-clamped at the indicated holding potentials and bathed in solutions containing different extracellular monovalent cations. The extracellular bathing solution was normal frog Ringer (filled diamonds), or a solution containing 1 mM MgCl₂; 2 mM HEPES, pH 7.4; with either 100 mM NaCl (squares), 100 mM KCl (circles), or 100 mM Tris, pH 7.4 (triangles). Data from a single representative cell are shown; similar results were obtained with several independent oocytes.

METHODS. PC12 cell poly(A)⁺ mRNA was isolated and a cDNA library constructed essentially as described²⁸. First-strand cDNA was synthesized using a NotI oligo(dT) primer; BstXI adapters were subsequently ligated onto the double-stranded cDNA. A directional library was constructed by inserting the resulting cDNA between the BstXI and NotI polylinker cloning sites of pcDNA1-Amp (Invitrogen). Plasmid DNA templates were linearized with NotI, transcribed with T7 RNA polymerase, and the resulting cRNA injected into oocytes²⁸. Voltage-clamp recordings were performed in a two electrode configuration (electrodes were filled with 3M KCl with resistances of 0.5–1.0 megohm). Recordings were performed in a continuous-flow chamber (perfusion rate, 1.0–1.5 ml min⁻¹). Current and voltage records were digitized at 2–20 Hz using MacLab data acquisition system.

likely to be multimeric complexes in which the lumen of the channel is lined by M2 domains from adjacent subunits. In oocytes, expression of a single cDNA clone is sufficient to produce fully functional receptor-ion-channel complexes, but this does not rule out the possibility that native P_{2X} receptors are heteromeric structures.

Extensive sequence similarity was found between $P_{2X}R1$ and a partial cDNA called RP-2 (Fig. 3a). RP-2 was isolated in a screen designed to identify mRNAs that are induced in immature rat thymocytes undergoing programmed cell death³. About 40% sequence identity is observed between $P_{2X}R1$ and RP-2. All of the cysteine residues in $P_{2X}R1$ are also found in RP-2, suggesting

a

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CAGCCCCGGCTTCCCGCGGGGCGGCCATGGTCCGCGCTTGGCCCGGGCTGTGGTCCGCTTCTGGGACTACGAGACGCTAAGTGATCGTGGTGCAGAAATCGGCGC 111
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CTGGGATTCGTGCACCGCATGGTGCAGCTTCTCATCTGCTTTACTTCTGTGTGTACGTCTTCATCGTGCAGAAAAGCTACCAGGACAGCGAGACCGGAGAGCTCC 222
LeuGlyPheValHisArgMetValGlnLeuIleLeuLeuTyrPheValTrpTyrValPheIleValGlnLysSerTyrGlnAspSerGluThrGlyProGluSerSer 65

M1
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AsnPheArgPheAlaLysTyrTyrLysIleAsnGlyThrThrThrThrArgThrLeuIleLysAlaTyrGlyIleArgIleAspValIleValHisGlyGlnAlaGlyLys 324

H5
TTCAGTCTCATTTCCACCATCATCAATCTGGCCACTGCTCTGACCTCCATCGGGTGGGCTCCTTCCTGTGTGACTGGATTTGTTAAGCTTATGAACAAAAACAAGCTC 1110
PheSerLeuIleProThrIleIleAsnLeuAlaThrAlaLeuThrSerIleGlyValGlySerPheLeuCysAspTrpIleLeuLeuThrPheMetAsnLysAsnLysLeu 361

M2
TACAGCCATAAGAAGTTCGACAAGGTGCGTACTCCAAGCATCCCTCAAGTAGATGGCCTGTGACCTTGCCTTGTCTTGGGCCAGATCCCTCCCCACCTAGTCACTAC 1221
TyrSerHisLysLysPheAspLysValArgThrProLysHisProSerSerArgTrpProValThrLeuAlaLeuValLeuGlyGlnIleProProProProSerHisTyr 398

TCCCAGGATCAGCCACCCAGCCCTCCATCAGGTGAAGGACCAACTTTGGGAGAAGGGGAGAGCTACCAGTGGTGTCCAGTCTCCTCGGCCCTTGCTCCATCTCTGCTCTG 1332
SerGlnAspGlnProProSerProProSerGlyGluGlyProThrLeuGlyGluGlyAlaGluLeuProLeuAlaValGlnSerProArgProCysSerIleSerAlaLeu 435

ACTGAGCAGGTGGTGACACACTTGGCCAGCATATGGGACAAAGACCTCTGTCCCTGAGCCTTCCCAACAGGACTCCACATCCACGACCCCAAGGTTTGGCCCCAACTT 1443
ThrGluGlnValValAspThrLeuGlyGlnHisMetGlyGlnArgProProValProGluProSerGlnGlnAspSerThrSerThrAspProLysGlyLeuAlaGlnLeu 472

TGATCTCATCTCTACTAACTACAGACCTGGACCTGGGAAGGCAGAGACAGCTTTGGCTGTGTAAGGCAGTCTAGAGAAGATCTGCGCTTTCAGTAACCATGTCCATGTG 1554
ACTGGGAAACAGAAACCTGTGCAAGAGGACAGGCGTCTTGTCTTAGCCCAAGCTTACATTCTCTCTCCTTAAGGCCTCTGGGAGAAGTGGGTTCCTTCCCATCTCCTT 1665
TCCCAACAGAACTCTCATAGGACCTTTCCTCTGCTACCTCTGTACTCTCATACAGTATTTCAGGAGACCCCAAGTTAGGGGCTATGCTCTGTGTATAATTTCAAGCCCC 1776
CCTTTAGAAGTTGACGATGCTGAGTTTCAATAAACAGTGATGAGC (A) -80 1822

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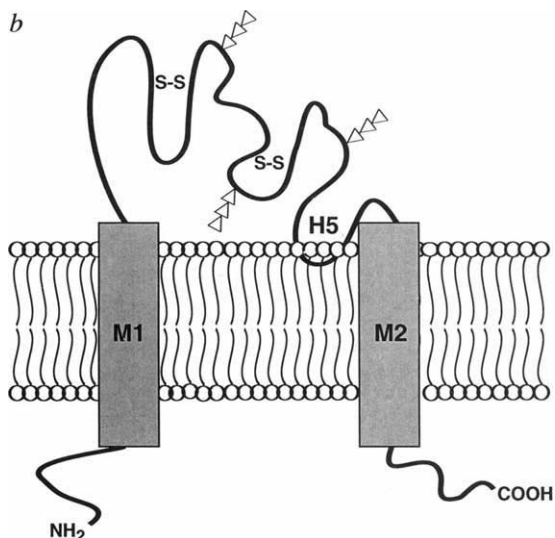


FIG. 2 The primary structure of the $P_{2X}R1$ ATP-gated ion channel. *a*, Nucleotide and deduced amino-acid sequence of $P_{2X}R1$. The deduced amino-acid sequence of 472 amino acids is displayed below the nucleotide sequence of the coding strand of the $P_{2X}R1$ cDNA. The initiator methionine was assigned to the first ATG in the cDNA sequence, which conforms well to the consensus translation initiation site. The potential transmembrane segments M1 and M2 are underlined, as is a segment resembling the H5 region of voltage-gated K^+ channels. Potential Walker type-A nucleotide-binding motifs²⁴ are shaded. Potential N-linked glycosylation sites are indicated by the arrowheads; cysteine residues in the proposed hydrophilic extracellular loop are indicated by asterisks. The proposed initiator methionine and consensus polyadenylation site are shown in bold. *b*, Model depicting a proposed transmembrane topology for $P_{2X}R1$. The $P_{2X}R1$ protein is shown with both N and C termini in the cytoplasm. Two putative membrane-spanning segments (M1 and M2) traverse the lipid bilayer of the plasma membrane and are connected by a hydrophilic segment of ~270 amino acids. This putative extracellular domain is shown containing two disulfide-bonded loops (S-S) and three N-linked glycosyl chains (triangles). The $P_{2X}R1$ cDNA was sequenced on both strands using Sequenase (USB). The Genbank accession number for this sequence is U14414.



FIG. 4 Tissue distribution of P_{2X}R1 mRNA. *a*, Northern blot analysis of P_{2X}R1 mRNA from adult rat tissues. Size markers are shown on the left ($M_r \times 10^{-3}$). *b*, *In situ* hybridization analysis of P_{2X}R1 mRNA expression in rat superior cervical ganglion (SCG). A dark-field photomicrograph showing hybridization with a ³⁵S-labelled antisense cRNA probe to a cryostat section taken through an adult rat SCG. Clusters of grains are seen over lightly staining cell bodies corresponding to presumptive neuronal cells. A 'sense' strand cRNA probe shows background grain density over a parallel tissue section.

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that they are functionally important. A helical wheel plot of the putative M2 segment of RP-2 closely resembles that of P_{2X}R1 (Fig. 3b), despite the limited sequence identity over this region. Although its function is presently unknown, RP-2 may encode an ion channel subunit that is activated by ATP or some other common intracellular metabolite released from dying cells.

A transcript of ~2 kilobases (kb) was detected by northern blot analysis in several tissues known to express P_{2X} receptors, including bladder, brain, spinal cord, intestine and vas deferens (Fig. 4a). The absence of detectable P_{2X}R1 RNA in skeletal muscle and heart, where functional ATP-gated channels have been found^{26,27}, supports the existence of distinct P_{2X} subtypes. Surprisingly, the most intense signal was seen with RNA from the pituitary, where a physiological role for extracellular ATP has not been described. *In situ* hybridization analysis of adult pituitary sections revealed a specific signal that was widely distributed over both intermediate and anterior lobes (not shown). There was no evidence for abundant expression by any particular cell type, consistent with the presence of P_{2X}R1 mRNA in a variety of neurosecretory cells and/or follicular stellate support cells. A more rigorous identification of pituitary cell types that express P_{2X}R1 will help to elucidate the role of ATP-gated ion channels in the neuroendocrine system. *In situ* hybridization analysis also showed expression of P_{2X}R1 transcripts within neurons of the superior cervical ganglion (Fig. 4b), consistent with the presence of ATP-gated ion channels in sensory ganglia¹⁶. Bright-field illumination of these sections reveals large, faintly staining cell bodies beneath the clusters of grains visible in dark field illumination.

Until now, all fast excitatory and inhibitory synaptic transmission was thought to be mediated by a well characterized superfamily of ligand-gated ion channels. Using the nicotinic acetylcholine receptor as a prototype, a model has been proposed in which five subunits, each consisting of a large N-terminal extracellular domain followed by four hydrophobic putative transmembrane segments, assemble to form a toroid, with residues from the second transmembrane α -helix of each subunit projecting into the ion pore. Agonists gate the channel by binding to a site(s) within the N-terminal extracellular domain (reviewed in ref. 23). Because ATP-gated ion channels are functionally quite similar to nicotinic acetylcholine receptors, it was expected that P_{2X} receptors would make use of the same molecular architecture to transduce fast excitatory responses. Instead, we have found that ATP-gated channels belong to an entirely different class of transmembrane signalling proteins. Members of this family include Na⁺, K⁺ and mechanosensory channels that monitor and regulate changes in cell volume, shape and membrane potential. The P_{2X} receptor has acquired the additional capacity to engage in intercellular communication by detecting the regulated (synaptic for example) or lytic (cell death for example) release of intracellular metabolites such as ATP. It remains to be seen whether signalling roles for other small endogenous molecules will be discovered through the identification of new members of P_{2X}/RP-2 receptor family. □

Received 24 June; accepted 31 August 1994.

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ACKNOWLEDGEMENTS. We thank F. Cohen, H. Ingraham, L. Jan, G. Lemke and R. Nicoll for discussion and comments on the manuscript, and L. Tecott and S. Dastmalchi for assistance with *in situ* hybridization experiments. This work was supported by the NIH and NIMH (Silvio Conte Center for Neuroscience), and by an NSF Presidential Young Investigator Award and a PEW Scholars Award in the Biomedical Sciences to D.J.

Yeast TAF_{II}s in a multisubunit complex required for activated transcription

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IN higher eukaryotes the RNA polymerase II transcription factor TFIID is composed of a TATA-box-binding protein (TBP) and a set of tightly bound polypeptides, designated TBP-associated factors (TAF_{II}s). One or more TAF_{II}s are coactivators that are required for activated but not basal transcription^{1–3}. The eukaryotic transcription machinery is highly conserved and it is therefore puzzling that TAF_{II}s have not been identified in yeast. Here we use TBP as a protein-affinity ligand to isolate from yeast a multisubunit complex that is required specifically for activated transcription by RNA polymerase II. Microsequence analysis and cloning of two subunits of this complex reveal that they are the homologues of known mammalian and *Drosophila* TAF_{II}s. The genes encoding these two yeast TAF_{II}s are essential, suggesting that activated transcription is required for viability of *Saccharomyces cerevisiae*.

To identify yeast coactivators that bind TBP, we used yeast TBP (yTBP) as the immobilized ligand for affinity chromatography. A yeast whole-cell extract was chromatographed on a control glutathione S-transferase (GST) or a GST–yTBP column. The flow-through fraction was tested for its ability to support transcription in the presence or absence of the activator GAL4–VP16. Figure 1 shows that the flow-through of the GST column supported both basal (lane 3) and activated (lane 4) transcription, but the flow-through of a GST–yTBP column supported only basal transcription (compare lanes 9 and 10). Addition of a 2M KCl eluate from the GST–yTBP column restored the ability of the GST–yTBP flow-through to respond to GAL4–VP16 (lane 14), whereas the eluate of the GST column had no effect (Fig. 1, and data not shown). The 2M KCl eluate from the GST–yTBP column had no transcriptional activity on its own (lanes 11 and 12) and did not significantly affect activator-independent basal transcription (lane 13). Taken together, these data identify a yeast coactivator activity that binds TBP.

The coactivator was purified from the GST–yTBP 2M KCl eluate as described in Fig. 2 legend. The polypeptide composition of the fractions with maximal coactivator activity from each column is shown in Fig. 2a. The final fraction from the hydroxyapatite column contained about eleven major polypeptides, with sizes ranging from 30K to 200K, and several minor peptides.

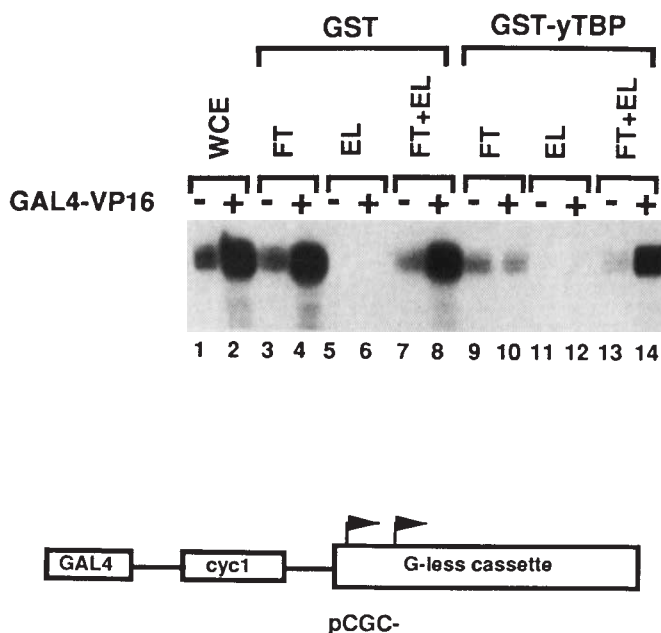


FIG. 1 Selective depletion of an essential coactivator on a GST-yTBP column. A yeast whole-cell extract (WCE) was diluted and chromatographed on a 2.5 ml GST or GST-yTBP column. Flow-through fractions (FT; 4 ml), peak 2M KCl eluates (EL; 1–2 ml), and an aliquot of unfractionated whole-cell extract (WCE) were collected and dialysed in parallel. Reactions contained the fractions indicated (8 μ l of WCE or column flow-through fractions and 5 μ l eluate fractions) and were performed in the presence (+) or absence (–) of 100 ng GAL4-VP16. A diagram of the DNA template¹⁵ is shown below the autoradiogram and contains the yeast *cyc1* promoter and a single GAL4-binding site upstream site of the TATA box.

METHODS. GST-yTBP and GST columns were prepared as described¹⁶, and protein loads were equalized to 1.5–2 mg bound fusion protein per ml of gel. The yeast GST-yTBP plasmid was constructed by cloning the *Bam*HI fragment from pASYIID¹⁷ into pGEX-1. A yeast WCE was prepared as described¹⁸ except that the ammonium sulphate pellet was dissolved and dialysed into buffer T containing 0.1 M potassium acetate. Buffer T is 20 mM HEPES-KOH, pH 7.6, 10 mM magnesium acetate, 5 mM EGTA, 5 mM DTT, 20% (v/v) glycerol plus protease inhibitors (0.5 μ g ml⁻¹ each of leupeptin, pepstatin A, aprotinin, antipain-HCl, chymostatin, bestatin; 2 mM benzamidin-HCl and 0.5 mM PMSF) and potassium acetate at the concentration indicated in parentheses. The WCE was diluted in buffer T (0.1 M) and passed four times over the columns followed by washing with 5 volumes of buffer T (0.2 M). Bound proteins were eluted with buffer T containing 2 M KCl for analytical runs. Eluates and flow-through fractions were dialysed into buffer T (0.1 M). Transcription reactions and sample processing were done as described¹⁸. GAL4-VP16 was prepared as described¹⁹, except that acetate buffers were used and the protein was purified further on heparin-agarose.

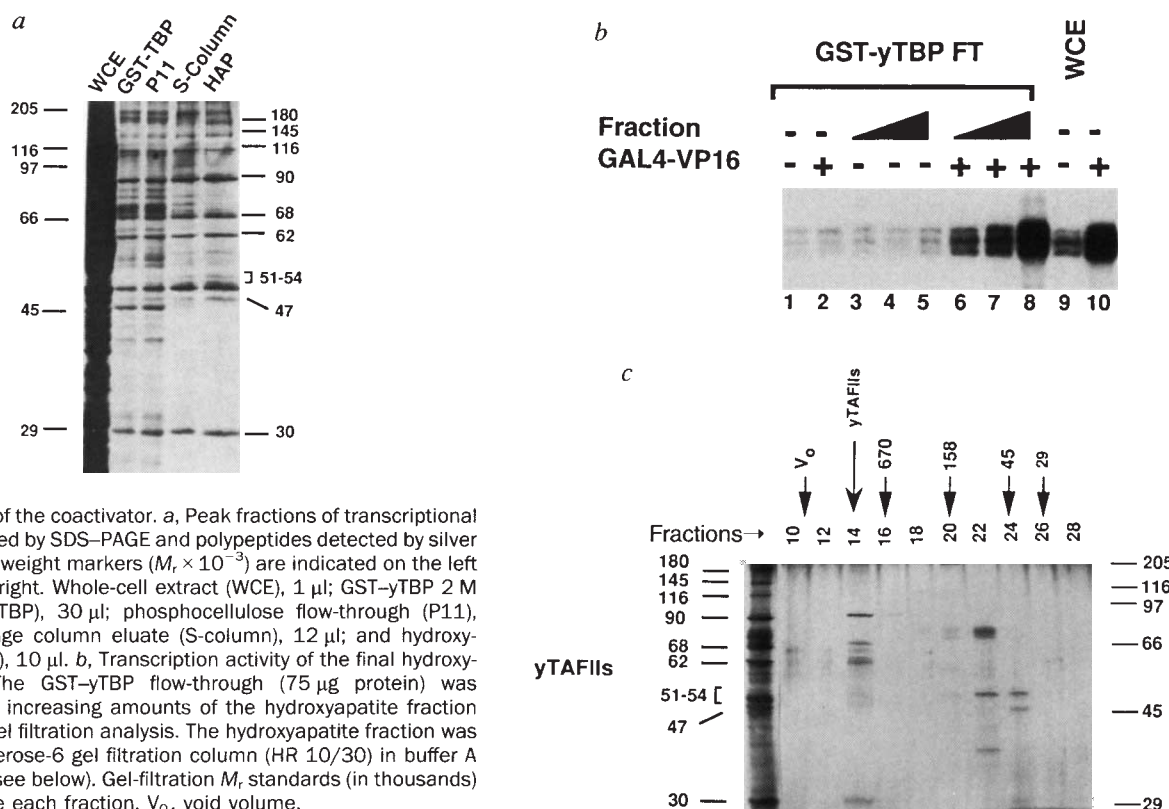


FIG. 2 Purification of the coactivator. *a*, Peak fractions of transcriptional activity were analysed by SDS-PAGE and polypeptides detected by silver staining. Molecular weight markers ($M_r \times 10^{-3}$) are indicated on the left and yTAFII_s on the right. Whole-cell extract (WCE), 1 μ l; GST-yTBP 2 M KCl eluates (GST-TBP), 30 μ l; phosphocellulose flow-through (P11), 35 μ l; S-ion-exchange column eluate (S-column), 12 μ l; and hydroxyapatite eluate (HAP), 10 μ l. *b*, Transcription activity of the final hydroxyapatite fraction. The GST-yTBP flow-through (75 μ g protein) was supplemented with increasing amounts of the hydroxyapatite fraction (~50–500 ng). *c*, Gel filtration analysis. The hydroxyapatite fraction was analysed on a Superose-6 gel filtration column (HR 10/30) in buffer A (0.3 M CH₃COOK) (see below). Gel-filtration M_r standards (in thousands) are indicated above each fraction. V_0 , void volume.

METHODS. WCE protein (~2 g) was passed 4–5 times over a 40-ml GST-TBP column in buffer T (0.1 M), the column was washed with 5 volumes of T (0.2 M) and eluted with step gradients of 0.5 M and 1.0 M CH₃COOK then 50 ml 2 M KCl with 0.003% NP-40, all in buffer T. Most of the activity eluted in the 2 M KCl fraction. Fractions were dialysed in buffer T (0.1 M) containing 0.003% NP-40. The peak activity from six columns (total WCE protein load 12 g, 800 ml) were pooled (9.5 mg, 180 ml) and passed over a 3 ml P11 column equilibrated in T (0.1 M). The flow-through fraction (8.6 mg, 185 ml) was loaded onto a 5 ml Econopak S-cartridge (BioRAD) equilibrated in buffer A (0.1). Buffer A is 20 mM HEPES-KOH, pH 7.6, 2 mM DTT, 1 mM EDTA, 0.003% (v/v)

NP-40, 20% (v/v) glycerol, with the molarity of potassium acetate indicated in parentheses. The S-column was washed and eluted with a linear gradient of A (0.1)–(0.5). Peak S-column fractions (2.1 mg, 36 ml) were dialysed in buffer A (0.1 M) and loaded onto a 1 ml Econopak hydroxyapatite column in buffer C (0.0 M potassium phosphate, pH 7.7, 0.2 mM EDTA, 2 mM DTT, 0.01% (v/v) NP-40, 20% (v/v) glycerol), washed, and eluted with a linear gradient to 0.3 M potassium phosphate. Peak fractions eluting between 0.15 and 0.30 M were pooled and dialysed into buffer A (0.1 M). About 1 mg (14 ml) protein was recovered after hydroxyapatite chromatography.

Nine of these (of sizes 180K, 145K, 116K, 90K, 68K, 62K, 51–54K, 47K and 30K) cofractionated with the transcriptional activity (Fig. 2a, and data not shown), and co-eluted on a Superose-6 gel filtration column (see later).

Figure 2b shows that addition of the final hydroxyapatite fraction to the GST-yTBP flow-through fraction restored, in a dose-dependent fashion, the ability of GAL4-VP16 to activate transcription (lanes 6–8). In contrast, equivalent amounts of this fraction had no effect on basal transcription activity (lanes 3–5).

The co-elution of these polypeptides over four chromatographic columns suggested that they might be components of a single complex. We therefore analysed the final hydroxyapatite fraction by gel-filtration chromatography. Figure 2c shows that on a Superose-6 gel-filtration column, nine of the polypeptides eluted as a native complex of relative molecular mass ~800K (fraction 14). Two of the eleven principal polypeptides contained in the hydroxyapatite fraction, M_s 200K and 50K, eluted separately in fractions 20 and 22–24, respectively. These two polypeptides also eluted slightly ahead of the complex on the hydroxyapatite column (data not shown). Figure 2c also reveals a cluster of polypeptides of 51–54K which eluted with the complex but were not well visualized in the hydroxyapatite fraction owing, at least in part, to masking by the 50K contaminant (Fig. 2a). This 51–54K cluster of polypeptides also co-immunoprecipitated with an anti-TBP antibody (see later) and we therefore believe that they are components of the complex. These com-

bined data indicate that the coactivator is contained within a complex of roughly nine polypeptides, which we refer to as the yeast TAF_{II} (yTAF_{II}) complex.

To confirm that the coactivator bound TBP, we used an anti-TBP antibody in co-immunoprecipitation experiments. Figure 3a shows that eight of the nine major polypeptides in the chromatographically purified yTAF_{II} complex were co-immunoprecipitated by this antibody and not by the control preimmune serum, the exception being yTAF_{II}116, which is relatively deficient in the immunoprecipitate compared to the hydroxyapatite fraction.

We next tested whether the immunoprecipitate contained coactivator activity. Immune complexes immobilized on protein A-agarose beads were washed with 2 M KCl, which released TBP-associated polypeptides but not TBP. Figure 3b shows that 2 M KCl wash of the immune complexes partially restored activated transcription to the GST-yTBP flow-through (lanes 11 and 12) without affecting the basal level (lanes 7 and 8). The reduced coactivator activity of the immune-complex eluate compared with the hydroxyapatite fraction (Figs 2b and 3b) is consistent with the 5–8-fold lower concentration of yTAF_{II}s in the immune-complex eluate (Fig. 3a).

Our results strongly suggested that one or more subunits of the yTAF_{II} complex bound TBP directly. To identify this subunit, we tested the yTAF_{II} complex for TBP binding using an immobilized protein blotting (far-western) assay. Figure 3c shows that a single subunit of the yTAF_{II} complex, the 145K

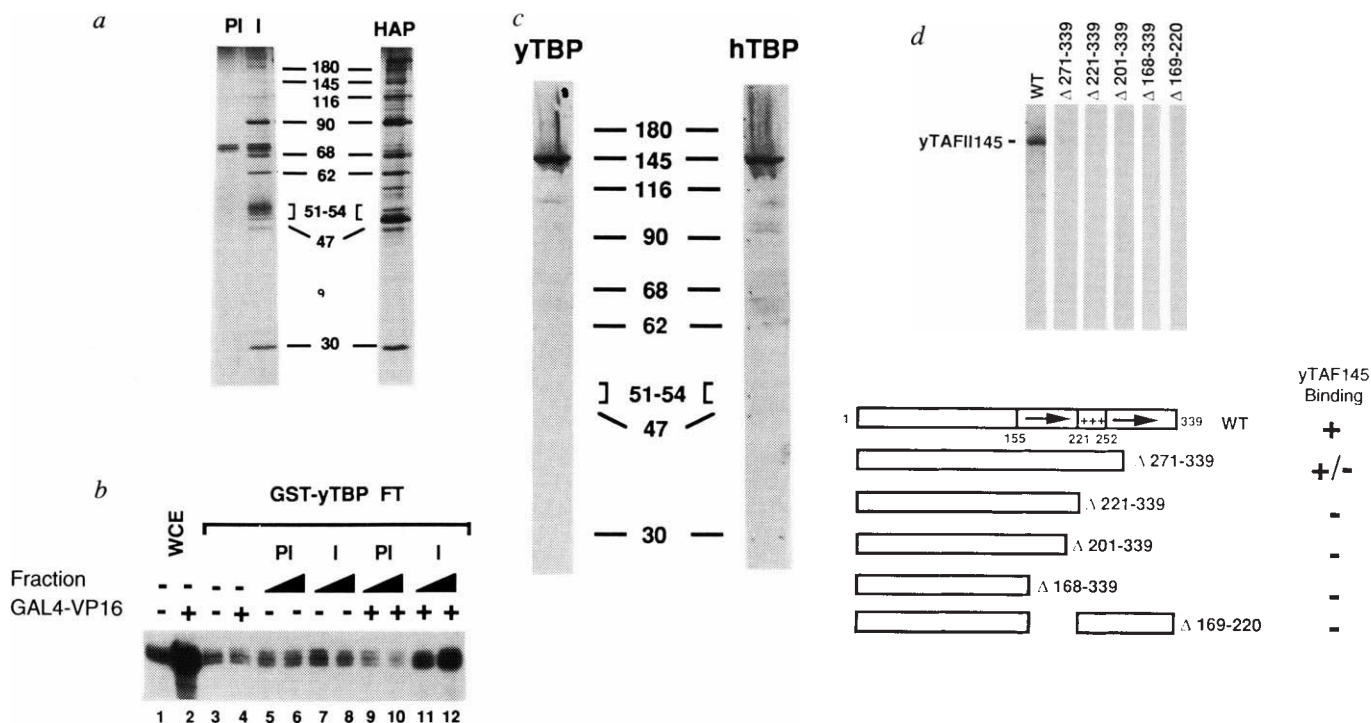


FIG. 3 Interaction of the coactivator with TBP. **a**, Co-immunoprecipitation of yTAF_{II}s from a yWCE by an anti-TBP antibody. The 2M-KCl eluate of the control preimmune (PI) and anti-TBP (I) immunoprecipitates, and the hydroxyapatite (HAP) fraction were analysed by SDS-PAGE and the polypeptides detected by silver staining. **b**, Co-immunoprecipitated yTAF_{II}s have coactivator activity. Aliquots of the indicated immune-complex eluates (~40 and 80 ng complex, estimated by silver staining) were added to reaction mixtures as described for Fig. 2b. **c**, Interaction between TBP and yTAF_{II}145. **d**, The region of TBP involved in the interaction with yTAF_{II}145.

METHODS. A yeast WCE was diluted and adjusted to 0.35 M CH₃COOK and NP-40 was added to 0.1% (v/v). 3-ml aliquots (40 mg ml⁻¹ protein) were incubated with affinity-purified preimmune or anti-TBP antibody (2 μg)¹⁴, incubated on ice for 5 h, then 10 μl protein A-agarose was

added and the incubation continued for 16 h with rotation at 4 °C. Beads were washed four times with 1 ml buffer T (0.9 M) containing 0.1% NP-40 (v/v). yTAF_{II}s were eluted by washes of buffer T (2 M KCl) plus 0.1% NP-40 (v/v) and 0.1 mg ml⁻¹ insulin, followed by dialysis into buffer T (0.15 M). For far-western analysis, membranes were blocked in buffer T (0.2 M) containing 0.01% (v/v) NP-40 and 4% BSA for 3 h at room temperature, then 2 h at 4 °C. ³⁵S-labelled yTBP and hTBP and its mutant derivatives were synthesized *in vitro*⁴ and were diluted in incubation buffer (buffer T (0.2 M) plus 0.01% (v/v) NP-40 and 0.5% BSA). The membrane was incubated with probe at 4 °C for 14–18 h, washed twice in incubation buffer for 15 min, twice in incubation buffer without BSA for 15 min, and rinsed in buffer T (0.2 M) without glycerol for 20 s.

b

yTAF₁₄₅ 1 MVKQCCSGKTNLANEAEYAEIPGEGFSGSIEGSIYIGGDEGA...NSKDYTEHLF.....DAVDFEDELADDDDLPE 72
 2 MEKSDSDGEGSIGNGLD.LTGILFGNIDS...EGRLLQDDGEGRGGTGFDAELENIGSLKGLDLSMLLEVDLKEAEPSPDDEE...E 87
 dTAF₂₅₀ 3

73 ESDANLHPAMTGMAYDDVN.....ENGAVIGDSNLSNMQLPENG...LSQQFILED.DGGTPATSNALFMGMANEIHLAT 148
 88 DARPSAVSAGSGHSFADALKAGVKREEREDGAVKAGQDAIDYSDITELSEDCPPTPESTSYDDLEDATPASKVEAKLTDDKEL.MPP 176
 149 ETGVLOGSGANEIGHSQSLSGVNGNDMSINGGFIMEPDMSDGKHKATKLDLINHEKYL...LKKYPPDFENGKILKNKL..... 227
 177 PSAPMRSGSGGLEEPKAS.....NDASSPSDDSKSTOSKADARLDTLPADILPSKYQNVDRLELFPDRFPQKVLRFSLRFGPGKPTS 260
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 288 SSASRGLIHVSIDEL.....FPIK.....EQKK...RKIIH...DEKTISED...LLIATDDMDQEKI..... 338
 350 VADWRFGAQIWDILEVPSGEGFVYGFKTKAASSTSSQQLKDERVKSPEDVDPSIADDAFLMVSQIHMEDVDVWDGNDIKAVLQ 439
 339 ...INQGSTSTATLADS.SMT.....PNIKF.....SGGYLK.....SLIEDVAD...WQMDMDMIDA. 387
 440 KLSKTHAGMLPSSGRTAGAFSQPKSMPVSGSSKQSGASSKKAQNAQAKPAEAPDDTWSLFFVNEELIYKMEDEVINDAQ 529
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 620 Y...TPKTEPT.LALKVGNLIQHSPTVVELRAFPVPTMGPMNVAFBRPPKKYSHGPMQASIPHPVPLKKTIA.KKAKQREVERIAS 705
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 614 LYNNMIRAPVFKEDISGDTLLTKSSGFGISNRYLRLNHLFVYQTPPEVEIPGPNRSKVTSMKATRLKMIYIRYL...NNHNSKASI 701
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 876 DDIKQAFPAHSESSIRKLRKQCADFKTGMDSNMVIKPEFRLPSEEEIRAMVSPECCAYFSMAIAEQLKDAQYGEKFLFAPQEDDE 965
 778 YNFDSKLSELE.NLLPMNITKFNINSTQHRAMIQIHGVGDTGCGEGTFLK.....TSMKGGF..... 836
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 1018LAGVTGKAARNKKG...NTTRCATCGQIGIRTNKSCPHYSKDNPAK 1066
 1321 GBSHEKRDGSKYKPSRKKPKPLDLKLCGACGQVGBMRTNACPLYSQMSLSQS 1379

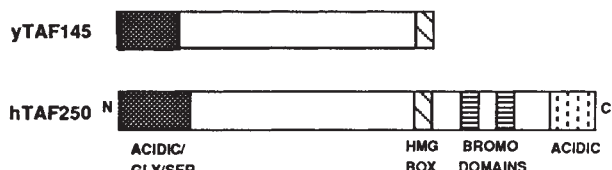


FIG. 4 Two yTAF₁₄₅s are the homologues of *Drosophila* TAF₁₄₅s and are essential for viability. *a*, Amino-acid sequence of yTAF₁₄₅ compared with dTAF₁₄₅. *b*, Amino-acid sequence of yTAF₁₄₅ and comparison to hTAF₂₅₀. The schematic drawing of hTAF₂₅₀ and identification of its purported domains are from ref. 9. The tryptic peptides obtained for each are underlined. *c*, Yeast TAF₁₄₅, and *d*, TAF₁₄₅ genes are required for viability. *S. cerevisiae* strain Cy246 was transformed with a disrupted copy of yTAF₁₄₅ (top centre in *c*) and yTAF₁₄₅ (top centre in *d*) to generate the diploid strains LY1 (TAF₁₄₅/taf₁₄₅:HIS3) and YSW57 (TAF₁₄₅/taf₁₄₅:LEU2), respectively. Southern blot analysis of genomic DNA isolated from the strains LY1 (digested with *Cla*I) and YSW57 (digested with *Eco*RV) confirms correct integration within the endogenous gene (bottom left in *c* and *d*). Both strains were sporulated and six randomly selected asci were dissected (bottom right in *c* and *d*).

METHODS. The hydroxyapatite fraction of yTAF₁₄₅ was used for microsequence analysis. A peptide with the sequence ATTEPSAEPDEPFIGYLGDVTA was obtained from yTAF₁₄₅. A BLAST search²⁰ of the database identified yTAF₁₄₅ as an unknown open reading frame on yeast chromosome III. yTAF₁₄₅ was cloned by PCR from genomic DNA. The peptides NINH-LFTVGQTFPVEEIPGN and LPVGETHVLGVQDKSPF were obtained from yTAF₁₄₅ and the degenerate oligonucleotides 5'-TT(C/T)CCNG-TNGA(A/G)GA(A/G)AT-3' and 5'-GTNGGNGA-(A/G)ACNCA(C/T)GT-3' were designed and used to screen a yeast genomic library²¹. The open reading frame was identified by sequencing and by its homology to *Drosophila* and human TAF₁₄₅. Disrupted copies of the yTAF₁₄₅ and yTAF₁₄₅ genes were constructed and genetic analysis was performed by standard methods²².

indicating that this complex is either TFIIB or is closely related to it. Although they may have some polypeptides in common, our yTAF₁₄₅ complex is probably a different complex for the following reasons. First, the GST-yTBP column failed to deplete the ability of an extract to support transcription by RNA polymerase III; conversely, addition of purified yTAF₁₄₅ complex to the GST-yTBP flow-through did not enhance transcription by RNA polymerase III (our unpublished data). Second, micro-

sequencing of yTAF₁₄₅ confirms that it is not BRF1/PCF4/TDS4 (our unpublished data). These combined results indicate that yeast contains at least two TBP-binding complexes, required for transcription by RNA polymerase III (ref. 14) and RNA polymerase II (this study).

The mechanisms by which TAF₁₄₅s and other coactivators facilitate activated transcription will become clearer as a result of the identification and cloning of yeast TAF₁₄₅s. □

Received 24 June; accepted August 1994.

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ACKNOWLEDGEMENTS. We thank D. Reinberg, R. Young, F. Winston, S. Hahn, N. Thompson and A. Weil for antibodies; R. Kornberg for plasmid pGCG; A. Berk for plasmid pYASII; M. Rose for the yeast library; W. Lane and the Harvard Microchemistry facility for microsequence analysis; C. Peterson for advice on yeast genetic methods and comments on the manuscript; M. Zapp and R. Singh and other members of our laboratory for comments on the manuscript; and L. Chang for technical assistance. This work was supported by grants from the NIH (M.R.G.), an NIH postdoctoral fellowship (S.S.W.) and a Damon Runyon-Walter Winchell Cancer Research Foundation Fellowship (J.C.R.).

Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding

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RETINOIC acid receptors (RARs) and retinoid X receptors (RXRs) regulate transcription by binding to response elements in target genes that generally consist of two direct repeat half-sites of consensus sequence AGGTCA (ref. 1). RAR/RXR heterodimers activate transcription in response to all-trans or 9-cis retinoic acid by binding to direct repeats spaced by five base pairs (DR5 elements)²⁻⁸, such that RAR occupies the downstream half-site⁹⁻¹². RXR homodimers activate transcription in response to 9-cis retinoic acid by binding to direct repeats spaced by one base pair (DR1 elements)^{8,13,14}. Although RXR/RAR heterodimers bind to DR1 elements with higher affinity than RXR homodimers, in most contexts they are unable to activate transcription in response to either all-trans or 9-cis retinoic acid³⁻⁵. As a result, RARs inhibit RXR-dependent transcription from these sites^{13,15}. We report that the switching of the RAR from an activator to an inhibitor of retinoid-dependent transcription requires that it be bound to the upstream half-site of DR1 elements and that it allosterically block the binding of ligand to the RXR.

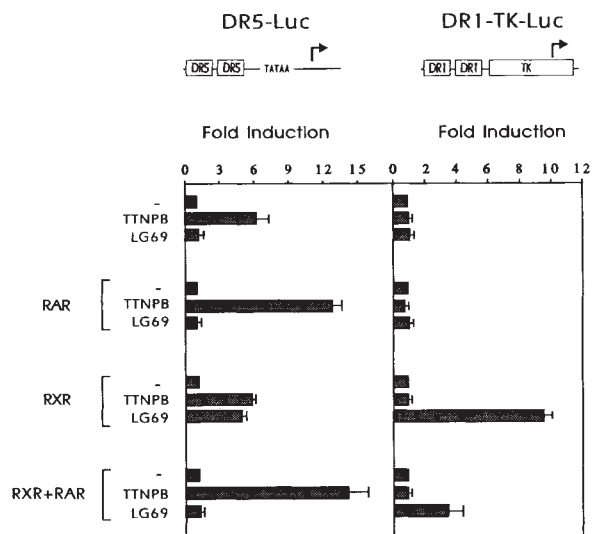
The specific contributions of the RAR and RXR to ligand-dependent transcription from promoters containing direct repeat elements spaced by 1 or 5 base pairs (bp), were determined in CV1 cells using the RAR-specific ligand TTNPB¹⁶, and the RXR-specific ligand LG69 (Fig. 1). In the absence of transfected receptors, TTNPB elicited a 13-fold transcriptional response

from the promoter containing DR5 elements, indicating the presence of functional endogenous receptors. Transfection of an RAR expression plasmid selectively increased the transcriptional response to TTNPB on the DR5-containing promoter, but did not lead to a transcriptional response from the DR1-containing promoter. Transfection of an RXR expression plasmid enabled LG69 to activate both promoters, with a preferential response from the DR1-containing promoter. These results confirmed the receptor specificities of each ligand, and illustrated the inability of the RAR to activate transcription from the DR1-containing promoter. When RAR was coexpressed with RXR, the transcriptional response to the RXR-specific ligand was significantly reduced from the DR1-containing promoter, whereas the response from the DR5-containing promoter was abolished, consistent with previous observations^{13,15}.

To determine whether these results reflected alterations in the ligand-binding properties of RARs and RXRs when bound to DNA, DNA-dependent ligand-binding assays were performed. The RAR-specific ligand, all-trans [³H]retinoic acid bound with high affinity to RAR/RXR heterodimers on both DR1 and DR5 elements (Fig. 2a) and was effectively competed by unlabelled all-trans retinoic acid and TTNPB, but not by LG69. The inability of the retinoic acid receptor to activate transcription from a DR1 element did not therefore reflect an inability to bind its activating ligand. As expected, 9-cis [³H]retinoic acid and [³H]LG69 bound with high affinity and specificity to RXR homodimers bound to the DR1 element (Fig. 2b). But with the addition of RAR and the formation of RAR/RXR heterodimers, binding of [³H]LG69 fell to near background levels (Fig. 2c). This result appeared paradoxical, because the addition of RAR dramatically increased RXR binding to the DR1 element, as demonstrated by parallel measurements of the binding of [³⁵S]RXR (data not shown). Similarly discordant binding of the RAR and RXR-specific ligands was observed for RAR/RXR heterodimers bound to the DR5 element (compare Fig. 2a and d), suggesting that the RAR prevented RXR from binding its ligand. This interpretation was supported by competition studies using the RAR and RXR-specific ligands. Although 9-cis retinoic acid binds to both RAR and RXR in solution ligand-binding assays^{8,14}, its binding to RAR/RXR heterodimers was competed for almost quantitatively by all-trans-retinoic acid and TTNPB on both the DR1 and DR5 elements (Fig. 2c and d). In contrast, LG69 was a much less effective competitor, indicating that 9-cis [³H]retinoic acid was preferentially, if not exclusively, bound to RAR. When binding of LG69 to RXR was carried out in solution, increasing molar ratios of RAR had no

FIG. 1 Effects of the RAR-specific ligand TTNPB, and the RXR-specific ligand LG69, on transcription from promoters containing DR1 and DR5 response elements. Promoter DR1-TK-Luc contains two copies of the DR1 element present in the cytosolic retinol-binding protein II promoter¹³ linked to the herpes simplex virus thymidine kinase promoter. DR5-Luc contains two copies of the DR5 element present within the RAR β 2 promoter²³⁻²⁵ linked to the RAR β 2 TATA and initiator sequences. Both promoters directed the expression of a luciferase cDNA. CV-1 cells were transfected with promoter plasmids and plasmids directing the expression of human RAR α (RSV-RAR) or human RXR α (CMV-RXR) as indicated. Cells were treated with 10⁻⁷ M TTNPB ((E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid) or LG69 (4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethenyl]benzoic acid) and assayed for luciferase activity.

METHODS. CV1 cells were transfected as previously described³. Each well received 1 μ g reporter plasmid and 100 ng of the indicated expression plasmid. Salmon sperm DNA was used to equalize the total quantity of transfected DNA. Error bars represent s.e.m.



effect (Fig. 2e). When assayed on a DR1 element, however, there was a progressive decrease in LG69 binding (Fig. 2f). This effect was independent of the order of addition of receptor, ligand and DNA (data not shown). The ability of RAR to inhibit ligand binding by RXR *in vitro* was closely correlated with its ability to inhibit transcriptional activation by RXR *in vivo* (Fig. 2f).

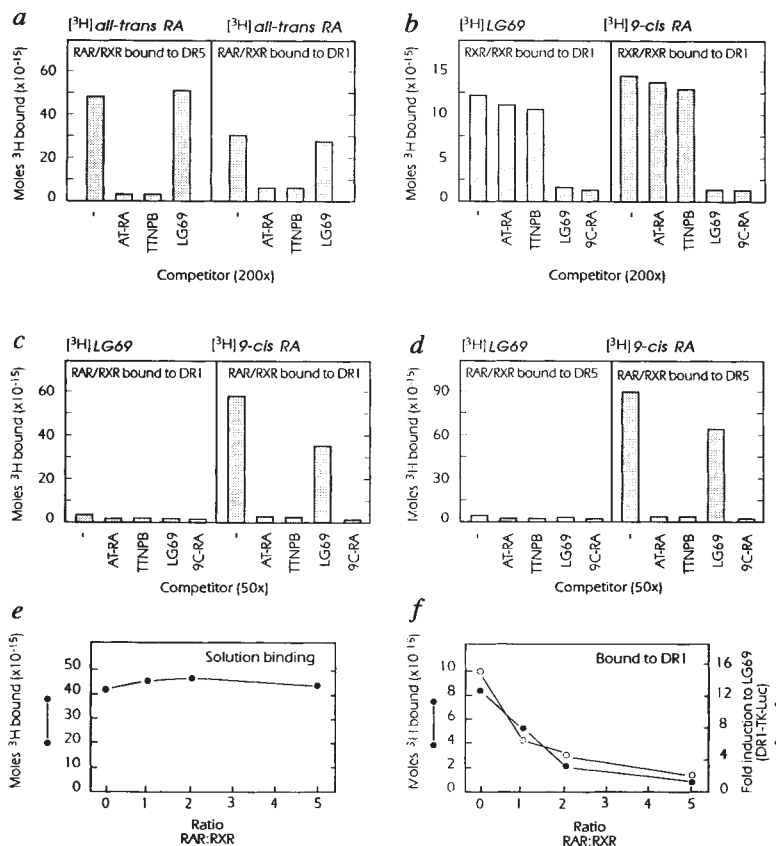
Although these observations provided an explanation for the inhibition of the transcriptional response to RXR-specific ligands by RAR, they did not account for the inability of RAR/RXR heterodimers to activate transcription from promoters containing DR1 elements in response to RAR-specific ligands. Because of the marked differences in the spatial relationships of half-sites spaced by 1 compared with 5 bp, RAR/RXR heterodimers might bind to a DR1 element in a conformation that prevented RAR from activating transcription. Direct repeat elements are inherently asymmetrical and in the case of DR5 elements, this asymmetry directs a specific polarity of heterodimer binding such that RXR binds to the upstream half-site and RAR to the downstream half-site^{9,12}. Using three independent approaches, RAR/RXR heterodimers were found to bind with an opposite polarity to DR1 elements, with the RAR located over the upstream half-site (Fig. 3). Polarity of binding was first suggested by experiments using an altered specificity mutant of RXR (RX-PGR, Fig. 4a) containing the P-box residues of the glucocorticoid receptor, which allow discrimination of the AGACA half-site that distinguishes glucocorticoid response elements (GRE) from the AGGTCA half-site recognized by RARs and RXRs^{17,19}. Heterodimers of RAR and RX-PGR bound to a DR1 element containing a downstream GRE half-

site (RIG) but not to a DR1 element containing an upstream GRE (GIR), indicating a requirement for RX-PGR to bind to the downstream half-site (Fig. 3b). This was confirmed by crosslinking experiments in which the photoreactive nucleotide iododeoxyuridine²⁰ was selectively introduced in place of thymidine within either the upstream or downstream half-sites of DR1 elements (Fig. 3c). In a third approach, the dimerization of a mutant RXR containing the T/A box of the RAR (RX-T/A, Fig. 3a) was examined. The T/A box lies immediately carboxy-terminal to the conserved DNA-binding domain and is required for cooperative binding of RXR homodimers to DR1 elements as it forms one side of an asymmetric dimerization interface^{12,21}. Structural studies of the RXR indicate that this region contains an α -helix that would be located over the 5' end of the half-site²². Mutation of this region would be predicted to impair cooperative binding of RAR/RXR heterodimers to direct repeat elements on which RXR bound to the downstream half-site, but not to response elements on which RXR bound to the upstream half-site. RX-T/A bound cooperatively with RAR on a direct repeat spaced by 5 bp, but not to one spaced by 1 bp (Fig. 3c), consistent with opposite polarities of binding on these two sites.

To test the possibility that the polarity of binding was itself a determinant of RAR function, we developed mutant receptors that enabled the ligand-binding domain of RAR to be specifically targeted to either the upstream or downstream half-sites of DR5 and DR1 elements. We constructed a chimaeric receptor (RX-RA, Fig. 3a) consisting of the amino terminus and DNA-binding domain of RXR fused to the ligand-binding

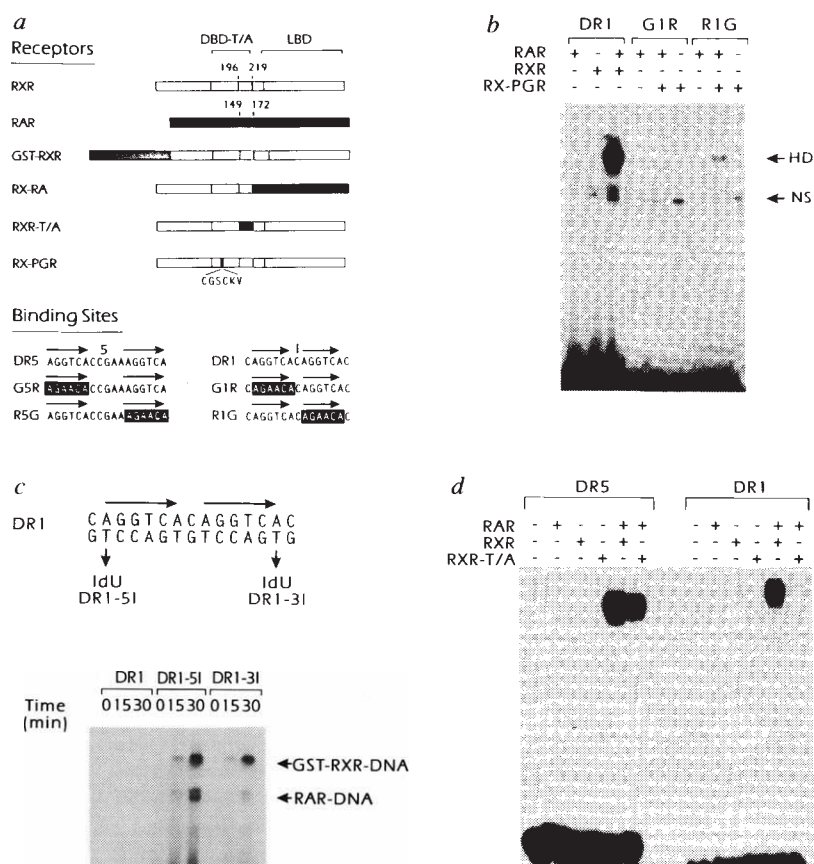
FIG. 2 Ligand-binding properties of RAR/RXR heterodimers and RXR homodimers bound to DNA. The indicated combinations of purified receptors were incubated with biotinylated oligonucleotides containing DR1 or DR5 elements. The predominant form of receptor binding (that is, homodimer versus heterodimer) was confirmed by parallel electrophoretic mobility shift assays. Incubations were performed in the presence of 20 nM concentrations of all-*trans* [³H]retinoic acid, 9-*cis* [³H]retinoic acid or [³H]LG69. **a**, RAR/RXR heterodimers bind the RAR-specific ligand all-*trans* retinoic acid with similar affinities on both DR1 and DR5 elements. **b**, RXR homodimers bind LG69 and 9-*cis* retinoic acid when bound to a DR1 element. **c**, RAR inhibits the binding of LG69 and 9-*cis* retinoic acid to RXR on a DR1 element. **d**, RAR inhibits the binding of LG69 and 9-*cis* retinoic acid to RXR on a DR5 element. **e**, Inhibition of the binding of LG69 to RXR by RAR does not occur in solution. Solution binding assays were performed as previously described⁸. **f**, Inhibition of the transcriptional response to LG69 on a DR1 element by RAR correlates with its ability to inhibit binding of LG69 to RXR. Binding of LG69 to RXR was determined in the absence and presence of the indicated mass ratios of RAR. The transcriptional response to LG69 was determined in CV1 cells using 100 ng RSV-RXR and the indicated mass ratios of RSV-RAR expression plasmids.

METHODS. RAR and RXR were synthesized in *E. coli* as glutathione-S-transferase fusion proteins, which were purified on a glutathione affinity matrix and the receptor portion eluted by cleavage with thrombin. Biotinylated oligonucleotides containing direct repeats spaced by 1 or 5 bp were prepared as previously described and gel-purified²⁶. To measure ligand binding to cognate receptors on specific DNA-binding sites, ~100 ng partially purified receptors and 50 ng biotinylated probe were incubated in 8 mM Tris-phosphate buffer (pH 7.4) containing 0.12 M KCl, 8% glycerol, 4 mM DTT, 0.1 mg ml⁻¹ poly(dI-dC) and 0.5% CHAPS detergent (Boehringer Mannheim) on ice for 30 min. Then, [³H]-labelled ligand (20–40 nM) was added to each reaction under dim light. Specific activities for the three radiolabelled ligands ranged between 28 and 56 Ci mmol⁻¹. Nonspecific binding was measured by



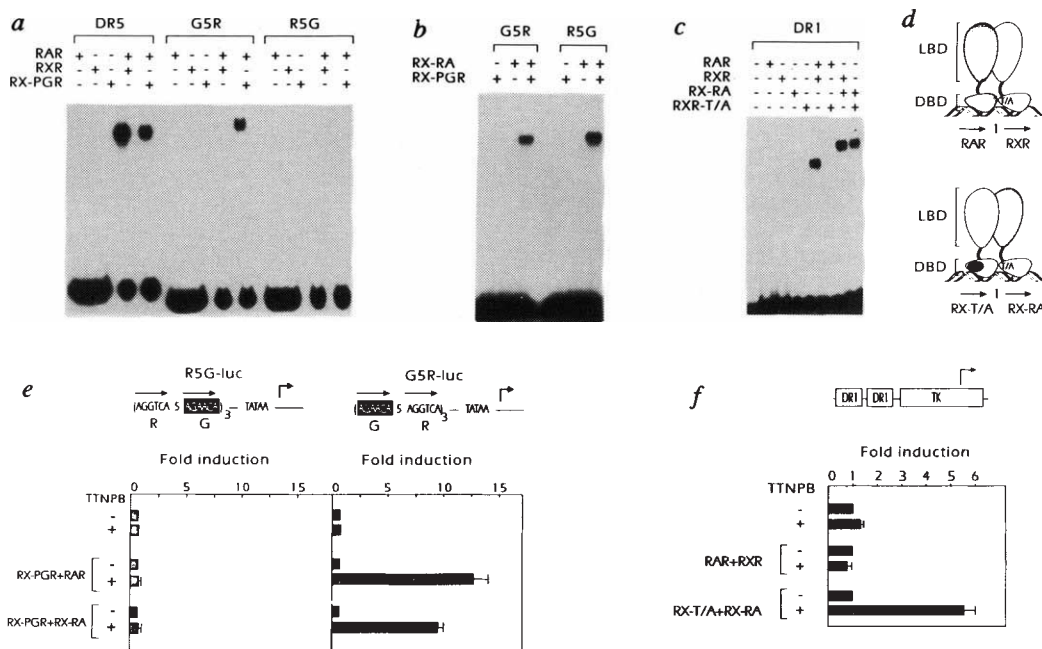
incubation with 50–200-fold excess molar of unlabelled ligand. After 16–18 h incubation at 4 °C, receptor-associated ligand was quantitated by precipitation with streptavidin–agarose as described²⁶. The results represent the mean values of duplicate points and are representative of more than six independent experiments.

FIG. 3 RAR binds to the upstream half-site of DR1 elements as a heterodimer with the RXR. **a**, Altered specificity receptors and DNA binding sites. The locations of the DNA-binding domain (DBD-T/A) and ligand binding/dimerization domain (LBD) of wild-type RARs and RXRs are illustrated. The sequence of the GRE half-site is indicated by reversed-out lettering. **b**, Binding of RAR and RX-PGR to G1R and R1G. Electrophoretic mobility shift assays were performed using *in vitro* translated proteins. Heterodimers of RX-RA and RX-PGR only bound to R1G, positioning RAR over the upstream half-site. **c**, Crosslinking of RAR to the upstream half-site of a DR1 element. Because the RAR and RXR are of similar size, a glutathione-S-transferase RXR (GST-RXR) fusion protein was used to allow it to be easily distinguished from the RAR on the basis of molecular weight. GST-RXR formed homodimers as well as heterodimers with RAR on the DR1 elements (data not shown), and could be crosslinked to both the upstream and downstream half-sites of the DR1 element in an iododeoxyuridine (IdU)-dependent manner. In contrast, IdU-dependent photocrosslinking of RAR was observed almost exclusively on the upstream half-site. **d**, Binding of RAR to DR1 and DR5 elements as a heterodimer with RXR or RX-T/A. Heterodimers of RAR and RX-T/A can bind with high affinity to the DR5 site, but not to the DR1 site. **METHODS.** RX-RA was generated using oligonucleotide-directed mutagenesis to introduce *Xho*I sites at the indicated junctions of RAR and RXR. Oligonucleotide-directed mutagenesis also generated RX-PGR, RA-PGR and RX-PGR-RA. These receptors contain P box residues from the glucocorticoid receptors required for high-affinity recognition of GRE half-sites¹⁷⁻¹⁹. Electrophoretic mobility shift assays were performed as previously described³. For crosslinking studies, the photoreactive nucleotide IdU replaced deoxythymidine at the indicated positions of a DR1 element (DR1-51 or DR1-31). Oligonucleotides selectively labelled in the antisense strand were incubated with RAR and GST-RXR, irradiated



with ultraviolet light (312 nm) for the indicated times and analysed by SDS-polyacrylamide gel electrophoresis.

FIG. 4 The polarity of binding is a determinant of the transcriptional response to RAR-specific ligands. **a**, Heterodimers of RAR and RX-PGR bind to the DR5 element containing an upstream GRE (G5R), but not to the DR5 element containing a downstream GRE (R5G), indicating a strict polarity of binding that positions the RAR over the downstream half-site. **b**, Heterodimers of RX-RA and RX-PGR bind to both G5R and R5G, indicating that RX-RA can be targeted to either the upstream or downstream half-site by RX-PGR and a downstream or upstream GRE, respectively. **c**, Heterodimers of RX-RA and RX-T/A bind to a DR1 element, whereas heterodimers of RAR and RX-T/A do not. **d**, Polarity of binding of RAR/RXR and RX-RA/RX-T/A heterodimers to the DR1 element. The RXR T/A box is required for binding to the downstream half-site. The RAR-T/A box mutation in RX-T/A (shaded ellipse in the DNA-binding domain) forces a polarity of binding with RX-RA in which RX-T/A is bound to the upstream half-site and RX-RA is bound to the downstream half-site. **e**, Heterodimers of RX-RA and RX-PGR respond to TTNPB only when RX-RA is targeted to the downstream



half-site of the G5R-containing promoter. **f**, Heterodimers of RX-RA and RX-T/A mediate a transcriptional response to TTNPB from a DR1-containing promoter, whereas heterodimers of RAR and RXR do not. Transient transfection experiments and electrophoretic mobility shift assays were performed as described in Figs 1 and 3, respectively.

domain of the RAR. Unlike the wild-type RAR, heterodimers of RX-RA and RX-PGR bound to G5R and R5G elements with roughly equivalent affinities (Fig. 4b), indicating that RX-RA could be targeted to either half-site of a DR5 element using the altered specificity mutant of the RXR (RX-PGR) and altered specificity binding sites containing downstream or upstream GRE half-sites. These properties of RX-RA permitted a direct assessment of whether the order of binding was a determinant of the transcriptional response to ligand. When targeted to the downstream half-site of a DR5 element (on the G5R-containing promoter) RX-RA conferred a significant transcriptional response to the RAR-specific ligand (Fig. 4c). When directed to the upstream half-site of a DR5 element (on the R5G-containing promoter), no transcription was observed, despite the fact that RX-RA and RX-PGR bound to both response elements with similar affinities.

We next examined the polarity of binding of RX-RA on DR1 elements by examining the ability of RX-RA to bind to DR1 elements as a heterodimer with the RXR chimera containing the T/A region of the RAR (RX-T/A). Unlike wild-type RAR, which could not heterodimerize with RX-T/A on the DR1 element owing to its requirement to bind to the upstream half-site, heterodimers of RX-RA and RX-T/A bound to the DR1 element with high affinity (Fig. 4c), indicating an order of binding in which RX-RA was bound to the downstream motif and RX-T/A to the upstream motif (Fig. 4d). In contrast to heterodimers of the wild-type RAR and RXR, heterodimers of RX-RA and RX-T/A conferred a marked transcriptional response to TNPB from the DR1-containing promoter (Fig. 4f). Together, these results provide strong evidence that the polarity of binding of RAR direct repeat elements is a determinant of its response to ligand.

These observations explain how the RAR inhibits retinoid-dependent transcription from DR1 elements by forming heterodimers with the RXR. First, heterodimerization inactivates the RXR by preventing ligand binding. Second, the transcriptional response of RAR to all-*trans* or 9-*cis* retinoic acid is prevented by its binding to the upstream half-site of DR1 elements. Regulation of gene expression by RXR in response to 9-*cis* retinoic acid must therefore occur independently of the RAR in order to escape the inhibitory effects of heterodimerization. These results also imply that RXR-specific ligands will regulate a different set of genes from RAR-specific ligands. Because

RAR/RXR heterodimers bind to DR1 elements with higher affinities than RXR homodimers, escape from the inhibitory effects of the RAR would require either marked increases in the expression of RXR, sufficient to drive the formation of homodimers, or the formation of heterodimeric associations between the RXR and other nuclear receptors that do not inhibit the response to 9-*cis* retinoic acid, such as the peroxisome proliferator activated receptors. Although the binding of a ligand is a special feature of nuclear receptors, the consequences of ligand interactions on protein structure can be achieved by other means, such as phosphorylation. The use of the inherent asymmetry of heterodimers as a mechanism of achieving complex transcriptional responses is therefore likely to extend beyond the nuclear receptor superfamily. □

Received 26 May; accepted 25 July 1994.

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ACKNOWLEDGEMENTS. R.K. and J.D.R. contributed equally to this work. We thank X.D. Fu and R. Stein for discussions, and M. Reyes and T. McQuistan for assistance in preparation of the manuscript. These studies were supported in part by grants from the NIH and the Lucille P. Markey Foundation. C.K.G. is a Lucille P. Markey Scholar.

Holliday junction cleavage by yeast Rad1 protein

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IN *Saccharomyces cerevisiae*, of the many genes required for excision repair of ultraviolet-damaged DNA, only *RAD1* and *RAD10* also function in genetic recombination¹. Complex formation between the *RAD1* and *RAD10* gene products¹ activates an endonucleolytic function that nicks single-stranded DNA^{2,3} and negatively supercoiled double-stranded DNA². To characterize the recombination role of the proteins Rad1 and Rad10, we have investigated their interaction with the Holliday junction, a four-stranded structure that results from single-stranded crossover between two duplex DNA molecules and whose resolution is obligatory for the generation of mature recombinants. We show that Rad1 binds

specifically to a Holliday junction and, in the presence of magnesium, catalyses the endonucleolytic cleavage of the junction. Junction cleavage by Rad1 proceeds sufficiently without Rad10, thus identifying Rad1 as the catalytic subunit of Rad1/Rad10 endonuclease.

As Rad1 and Rad10 form a tight physical complex⁴, to avoid co-elution of Rad10 with Rad1 when purifying the latter, we used the *rad10* genomic deletion strain YR10-1 as host for the Rad1-overproducing plasmid pRR168² (2 μ replication origin, *ADC1::RAD1*). Rad1, relative molecular mass 125,000 (M_r 125K) was purified from extract of YR10-1[pRR168] to homogeneity (Fig. 1a, left panel) using our published procedure². Rad10 (M_r 24,500) was purified to homogeneity (Fig. 1a, right panel) from the *RAD+* yeast strain CMY135 harbouring the Rad10-overproducing plasmid pSUC8 (2 μ , *ADC1::RAD10*) as described previously^{2,5}. In purifying Rad10, we did not encounter any problem with co-purification of Rad1, because the chromatographic procedure we used, which includes a molecular sizing step^{2,5}, selects for uncomplexed Rad10.

During recombination of two duplex DNA molecules, a symmetrical four-stranded structure termed the Holliday junction is formed, the resolution of which yields mature recombinants⁶. To study the interaction of Rad1 and Rad10 with the Holliday junction, we constructed a synthetic junction by hybridizing four

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oligonucleotides, 49–51 bases long as described⁷. The junction DNA, referred to as X12, contains a central core region with 12 base pairs (bp) of homology⁷ (see Fig. 2b). The presence of heterologous sequences in the four arms of X12 limits spontaneous branch migration to the central homologous core only, helping to preserve the junction structure. As shown in Fig. 1b, incubation of ³²P-labelled X12 junction DNA (1 ng, 3 pmol nucleotides) with Rad1 (10–50 ng, 0.08–0.4 pmol) results in the formation of an X12 species (lanes 8–12) with retarded mobility in polyacrylamide gels, indicating complex formation between X12 DNA and Rad1. The amount of Rad1–X12 complex increases with Rad1 protein concentration (Fig. 1b and c), and complex formation does not require Mg²⁺ (Fig. 1b) or ATP (data not shown). By contrast, a ³²P-labelled DNA duplex, obtained by hybridizing one of the oligonucleotides used in X12 construction to its exact complement (see Fig. 1 legend), is not bound by Rad1 at 10 and 20 ng of protein (Fig. 1b, lanes 2 and 3; Fig. 1c), and only a low level of complex formation with Rad1 was detected at the higher protein concentrations (Fig. 1b,

lanes 4–6; Fig. 1c). These observations indicate that Rad1 binds specifically to the Holliday structure. In agreement with these observations, we find that binding of Rad1 to the Holliday junction DNA is not competed away by a 100-fold excess of duplex DNA.

In the presence of Mg²⁺, Rad1 acts on the X12 junction to yield products with the same electrophoretic mobility as duplex DNA (Fig. 1d). The yield of duplex product increases as a function of Rad1 protein concentration (Fig. 1d, lanes 5–8), requires Mg²⁺ (Fig. 1e, lane 3), but shows no dependence on ATP (data not shown). In the same incubation conditions, no alteration of the mobility of the control DNA duplex was observed (Fig. 1d, lanes 1–4). The junction-resolving activity is strongly inhibited by anti-Rad1 antibodies⁴ (Fig. 1e, lane 5), but not by antibodies specific for the Rad2 (ref. 4), Rad10 (ref. 4), or Rad23 (ref. 8) (Fig. 1e, lanes 6–8). This result, coupled with the high purity of our Rad1 (see Fig. 1a, left panel), provides strong evidence that the Holliday junction-resolving activity resides in the Rad1 protein.

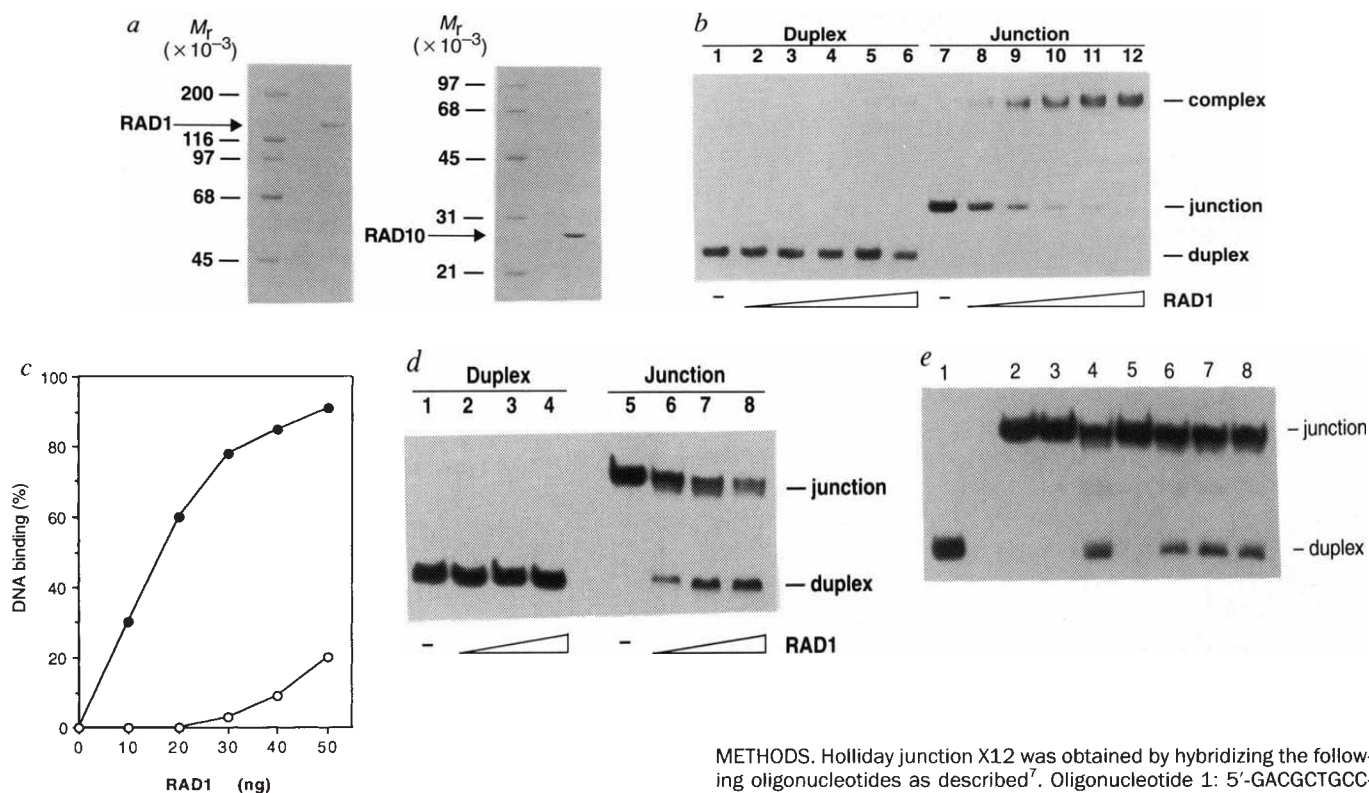
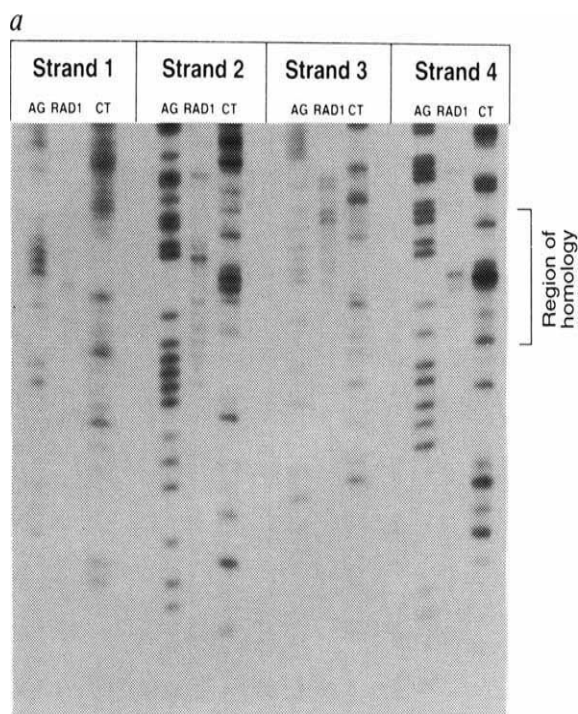


FIG. 1 Rad1 protein binds and resolves the Holliday structure. **a**, SDS-PAGE analysis of Rad1 and Rad10. Proteins (1 µg each) were fractionated in 7% (Rad1) and 10% (Rad10) denaturing polyacrylamide gels and stained with Coomassie blue. **b**, Specific binding of RAD1 protein to a synthetic Holliday junction. Duplex DNA (lanes 1–6) and the synthetic Holliday junction X12 (lanes 7–12), 0.5 and 1 ng respectively, were incubated alone or with 10 ng (lanes 2, 8), 20 ng (lanes 3, 9), 30 ng (lanes 4, 10), 40 ng (lanes 5, 11) and 50 ng (lanes 6, 12) Rad1 protein in the absence of Mg²⁺ at 10 °C for 30 min. **c**, The autoradiogram in **b** was scanned with a densitometer to obtain data points for a graphical representation of the results. **d**, Rad1 resolves X12 to yield duplex products. Duplex DNA (0.5 ng, lanes 1–4) and X12 junction DNA (1 ng, lanes 5–8), were incubated with 20 ng (lanes 2, 6), 30 ng (lanes 3, 7) and 40 ng (lanes 4, 8) of Rad1 in the presence of Mg²⁺ for 15 min at 30 °C. **e**, Junction resolution is specifically inhibited by anti-Rad1 antibodies. X12 junction DNA (1 ng) was incubated for 15 min at 30 °C alone (lane 2) or with 35 ng of Rad1 (lanes 3–8) in the absence of Mg²⁺ (lane 3) and in the presence of Mg²⁺ (lanes 4–8). One of the following affinity-purified polyclonal antibody preparations (400 ng protein) were included in the reaction mixtures in lanes 5 to 8: anti-Rad1 (lane 5), anti-Rad2 (lane 6), anti-Rad10 (lane 7) and anti-Rad23 (lane 8). Duplex DNA (lane 1) was included in this analysis as marker.

METHODS. Holliday junction X12 was obtained by hybridizing the following oligonucleotides as described⁷. Oligonucleotide 1: 5'-GACGCTGCC-GAATTCCTGGCTTGTAGGACATCTTTGCCACGTTGACCC-3'. Oligonucleotide 2: 5'-TGGGTCAACGTGGGCAAGATGTCCTAGCAATGTAATCGTCTATG-ACGTT-3'. Oligonucleotide 3: 5'-CAACGTCATAGACGATTACATTGCTA-GGACATGCTGTCTAGAGACTATCGA-3'. Oligonucleotide 4: 5'-ATCGATAGT-CTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGCGT-3'. Oligonucleotide 1 was labelled with ³²P at the 5' end using T4 polynucleotide kinase. The junction DNA contained a central homologous core of 12 bp (underlined). Duplex DNA used was constructed by hybridizing ³²P-labelled oligonucleotide 1 to its exact complement (not shown). To examine the binding of duplex and junction DNAs, 0.5 ng and 1 ng respectively (4,000 c.p.m. total) of DNA was incubated with increasing amounts of Rad1 protein (10–50 ng) in 10 µl of buffer A (50 mM Tris-HCl, pH 8.0, containing 100 µg ml⁻¹ BSA, 50 mM KCl, 1 mM DTT and 0.5 mM EDTA). After 30 min at 10 °C, the reaction mixtures were subjected to electrophoresis (25 mA for 1 h) at 4 °C in 0.75-mm thick 4% polyacrylamide gels in 40 mM Tris-acetate, pH 7.4, containing 2 mM EDTA. For X12 junction resolution, 1 ng of DNA was incubated with Rad1 protein (20–40 ng) at 30 °C for 15 min in 10 µl buffer B (Buffer A containing 5 mM MgCl₂) and the reaction was terminated by adding 1.2 µl 5% SDS and 1 µl of proteinase K (10 mg ml⁻¹ stock). After a 10 min incubation at 37 °C, reaction mixtures were subjected to electrophoresis (12.5 mA for 1 h) in 0.75-mm-thick 8% polyacrylamide gels at 4 °C in 90 mM Tris-borate, pH 8.0, containing 2 mM EDTA.



To determine the mechanism of junction resolution, four separate preparations of X12, each labelled with ^{32}P at the 5' end in DNA strand 1, 2, 3 or 4, were incubated with Rad1 and Mg^{2+} , and the reaction products were analysed in a denaturing polyacrylamide gel with A+G and C+T sequence ladders. Figure 2a shows that Rad1 nicks the DNA strands in the junction DNA as schematically illustrated in Fig. 2b, which shows the central core and adjoining region, with the incision sites in the four constituent oligonucleotides. The multiplicity of cleavage sites could arise as a result of branch migration of the junction. We suggest that the duplex-sized DNA product (Fig. 1d and e) is generated as a result of nicking by Rad1 of two DNA strands of like polarity in the Holliday structure. The small amount of DNA migrating between the junction and duplex DNA (Fig. 1d) may correspond to a three-armed structure formed by aberrant nicking in DNA strands of opposite polarity.

To examine the effect on Holliday junction binding and cleavage of removing homology from the central core, we prepared another synthetic junction that lacks homology in the core region (see Fig. 3 legend); this junction structure is referred to as X0. In the absence of Mg^{2+} , essentially the same proportions of junction X0 and junction X12 were bound at three different

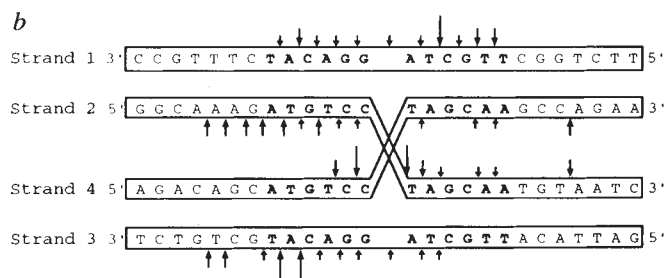


FIG. 2 X12 junction resolution is mediated by endonucleolytic cleavage of the DNA strands. **a**, Four different preparations of X12 (1 ng each) differing only in the DNA strand that was labelled with ^{32}P were incubated in the presence of Mg^{2+} with 30 ng Rad1 at 30 °C for 15 min. The reaction was stopped by the addition of an equal volume of 50 mM Tris-HCl, pH 8.0, containing 80% (v/v) formamide, 1 mM EDTA, 0.1% (w/v) xylene cyanol and 0.1% bromophenol blue, heated at 95 °C for 3 min, and then chilled on ice for 5 min. Samples were loaded onto a 12% polyacrylamide gel made in Tris-borate, EDTA containing 7 M urea, and electrophoresed for 3 h at 12 mA and 20 °C. The gel was soaked for 10 min in 5% each of methanol and acetic acid, dried, and subjected to autoradiography. G+A and C+T sequence ladders were obtained by chemical cleavage according to Maxam and Gilbert, using a kit purchased from Dupont. **b**, Diagram showing the cut sites in the X12 junction DNA. Bases constituting the homologous core are shown in bold type and arrows indicate the cut sides.

amounts of Rad1 protein (Fig. 3a). When Mg^{2+} was present, junction X12 was resolved to yield duplex products (Fig. 3b, lanes 6–9), but in contrast, there was only a hint of X0 resolution at the highest amount of Rad1 protein used (Fig. 3b, lanes 2–5). These results suggest that binding and cleavage of the Holliday structure by Rad1 are two distinct, separable events, with the cleavage step being stimulated by the presence of sequence homology in the structure. The duplex DNA formed from resolution of X0 junction (Fig. 3b, lane 5) arises from nicks made in strands of the same polarity between seven and nine nucleotides away from the crossover point (data not shown).

As Rad1 and Rad10 proteins together comprise a single-stranded endonuclease (Fig. 4a), we investigated whether Rad10 protein would increase the efficiency of Rad1-catalysed junction cleavage. Rad10 does not act on duplex DNA or on junction X12 (Fig. 4b). The addition of Rad10 to reaction mixtures containing increasing amounts of Rad1 did not affect the amount of duplex product generated as a result of X12 resolution. The pattern of nicking of the four strands of X12 junction was the same with the mixture of Rad1 and Rad10 proteins as with Rad1 alone (data not shown).

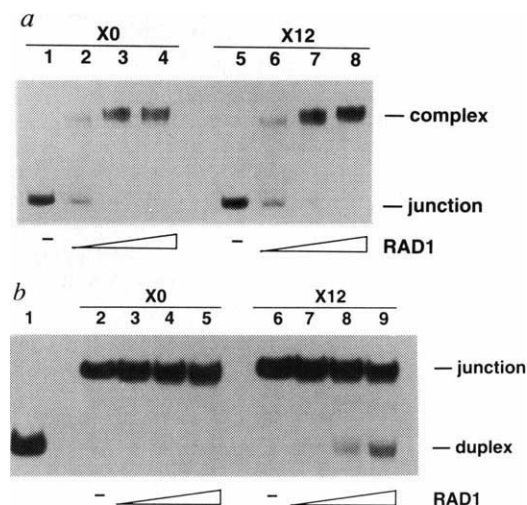


FIG. 3 Junction resolution is stimulated by homology. **a**, Rad1 binds junction DNA without a homologous core as efficiently as junction X12. Junctions X0 (lanes 1–4) and X12 (lanes 5–8) (1 ng each) were incubated in the absence of Mg^{2+} with 15 ng (lanes 2, 6), 30 ng (lanes 3, 7), and 45 ng (lanes 4, 8) Rad1 protein at 10 °C for 30 min. Nucleoprotein complexes were detected as described in Fig. 1 legend. **b**, Junction X12 is resolved preferentially. X0 (lanes 2–5) and X12 (lanes 6–9) (1 ng each) were incubated in the presence of Mg^{2+} with 10 ng (lanes 3, 7), 20 ng (lanes 4, 8) and 30 ng (lanes 5, 9) of Rad1 for 15 min at 30 °C. Duplex DNA (lane 1). Products of junction resolution were identified as described in Fig. 1 legend.

METHODS. Junction X0, which differs from X12 in lacking a homologous core, was obtained by hybridizing the following oligonucleotides; oligonucleotide 1 was 5' end-labelled with ^{32}P before hybridization. Oligonucleotide 1: 5'-GACGCTGCCGAATTCTGGCGTTAGGAGATACCGATAAGC-TTCGGCTTAA-3'. Oligonucleotide 2: 5'-CTTAAGCCGAAGCTTATCGGTATC-TTGCTACGACGCTAGCAAGTGATC-3'. Oligonucleotide 3: 5'-TGATCA-CTTGCTAGCGCTCGTAAGCAGCTCGTGCTGTCTAGAGACATCGA-3'. Oligonucleotide 4: 5'-ATCGATGTCTCTAGACAGCACGAGCCCTAACGCCGAATT-CGGCAGCGT-3'. Junction X0 was purified and used in binding and resolution experiments as described for junction X12 in Fig. 1.

FIG. 4 Rad10 protein is required for cleavage of single-stranded (ss) DNA but has no effect on Holliday junction cleavage. *a*, Rad1–Rad10 nuclease action on ssDNA. Viral circular ssM13 DNA (200 ng, lanes 1–4) was incubated in buffer B (see Fig. 1 legend) with 150 ng Rad1 (lane 2), with 50 ng Rad10 (lane 3), or with 150 ng Rad1 and 50 ng Rad10 together (lane 4) for 20 min at 30 °C. Reaction mixtures were deproteinized and analysed in a 0.8% agarose gel as described². *b*, Rad10 does not affect Holliday junction cleavage. Duplex DNA (lanes 1–4) and X12 junction DNA (lanes 5–10), 0.5 ng and 1 ng respectively, were incubated with RAD1 (lanes 3, 7, 8), with RAD10 (lanes 2, 6), or with both proteins together (lanes 4, 9, 10) for 15 min at 30 °C and analysed. In the last



In *Escherichia coli*, the product of *RuvC* resolves the Holliday structure through DNA strand cleavage^{7,9}. But whereas Rad1 incises at multiple sites in the junction DNA without any strong sequence preference, endonucleolytic scission of junction DNA by *RuvC* protein shows highly restricted specificity as it occurs efficiently only at the 3' side of thymine residues¹⁰. Furthermore, *RuvC* introduces symmetrically related nicks in junction DNA¹⁰, whereas Rad1 nicks junction DNA at multiple sites, many of which are asymmetrical.

Our finding that Rad1 cleaves Holliday junctions agrees with genetic studies indicating that the *RAD1* and *RAD10* counterparts in the fission yeast *Schizosaccharomyces pombe* function in the resolution of recombination intermediates during mating-type switching. Of the ten *swi* genes involved in mating-type switching in *S. pombe*, *swi4*, *swi8*, *swi9* and *swi10* affect the resolution of recombination intermediates, and because of aberrant resolution, mutants of these genes show increased incidence of rearrangements of the mating-type locus¹¹. *swi9* and *swi10* are the respective *S. pombe* homologues of *S. cerevisiae* *RAD1* and *RAD10* genes^{12,13}. Even though Rad1 alone cleaves Holliday junction, it introduces many asymmetrical nicks in the junction DNA. We suggest that a complex consisting of Rad1, Rad10

case (lanes 4, 9, 10), Rad1 and Rad10 were mixed together and incubated for 15 min on ice before incubation with DNA. Either 15 ng (lanes 7, 9) or 30 ng (lanes 3, 4, 8, 10) Rad1 and 10 ng of Rad10 (lanes 2, 4, 6, 9, 10) were used.

and the *S. cerevisiae* counterparts of the *swi4* and *swi8* proteins would resolve Holliday junction by nicking strands of the same polarity in a symmetric fashion. However, it is also possible that some of these other proteins function in other phases of recombination⁵. □

Received 2 June; accepted 17 August 1994.

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ACKNOWLEDGEMENTS. This work was supported by the US National Cancer Institute and the Department of Energy.

Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair

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In mammalian cells, DNA damage increases the levels of the nuclear tumour-suppressor p53, resulting in elevated synthesis of p21, an inhibitor of cyclin-dependent kinases (CDK)^{1–6}. p21 may also directly block DNA replication by inhibiting the proliferating-cell nuclear antigen (PCNA)⁷, an essential DNA replication protein. However, PCNA is also required for nucleotide-excision repair of DNA⁸, an intrinsic part of the cellular response to ultraviolet irradiation. Using an *in vitro* system⁹, we now show that p21 does not block PCNA-dependent nucleotide-excision repair, in contrast to its inhibition of simian virus 40 DNA replication⁷. Furthermore, the short gap-filling DNA synthesis by PCNA-dependent DNA polymerases δ and ϵ is less sensitive to inhibition by p21 than is long primer-extension synthesis. The ability of p21 to inhibit the role of PCNA in DNA replication but not in DNA repair rationalizes *in vivo* data showing that genetic damage leads

to inactivation of chromosomal replication while allowing damage-responsive repair.

To compare the effect of p21 on DNA repair and replication, whole-cell extracts were prepared from RKO cells, a human colorectal carcinoma cell line containing wild-type p53 (ref. 10). Following published procedures⁹, we showed that the RKO cell extract preferentially repaired ultraviolet-damaged plasmid DNA templates in the presence of undamaged control DNA (Fig. 1a, lanes 1 and 2). The same extract also supports large-T-antigen-dependent replication of plasmids containing the simian virus 40 (SV40) replication origin (Fig. 1a, lanes 5 and 6). As shown previously⁷, purified recombinant p21 abolished SV40 DNA replication *in vitro* (Fig. 1a, lanes 7 and 8; see Fig. 1b for quantification). Addition of purified PCNA overcame the p21 inhibition of DNA replication (ref. 7, and data not shown). In contrast, similar amounts of p21 did not affect DNA repair *in vitro* (Fig. 1a, lanes 3 and 4, and Fig. 1b for quantification). To verify that this result is not restricted to extracts from tumour cell lines, we prepared extracts from a cell line (GM02184) derived from healthy human lymphoblasts. As shown in Fig. 2, p21 again blocked SV40 DNA replication but was without effect on DNA repair. These *in vitro* results are consistent with data from ultraviolet-irradiated cells in which replicative DNA synthesis as well as cell-cycle progression is inhibited, yet DNA repair persists^{11,12}.

To ascertain the dependence of the *in vitro* repair system on PCNA, we fractionated the RKO cell extract so that reconstituted DNA repair depended on purified PCNA, replication protein A and a fractionated extract (fraction II) (Fig. 3, lanes 4 and 5; see also ref. 8). Replication protein A is a cellular single-

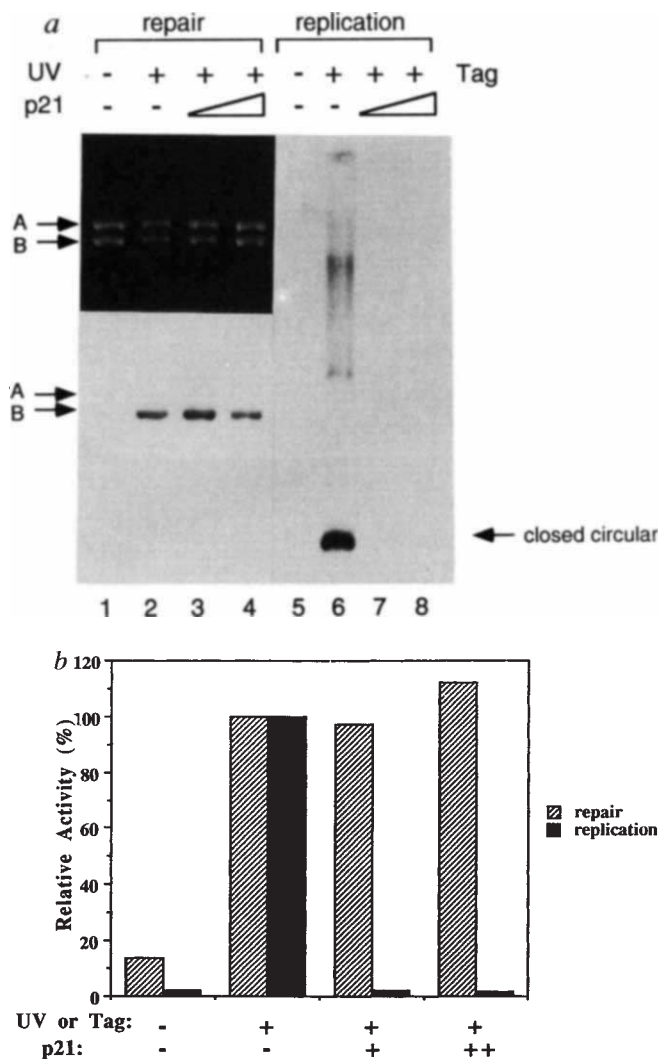


FIG. 1. p21 inhibits SV40 DNA replication, but not DNA repair. *a*, DNA repair (lanes 1–4) and replication (lanes 5–8) in an RKO cell extract. The top and bottom panels in lanes 1–4 show a photograph and an autoradiogram of the same gel. Arrows A and B represent pKS0 (ref. 26), a non-irradiated plasmid and pSV011 (ref. 27), a UV-irradiated plasmid. Neither plasmid in lane 1 was irradiated. DNA replication was carried out in either the absence (lane 5) or presence of the SV40 large T-antigen (lanes 6–8). Replication and linearized repair products were analysed by agarose gel electrophoresis in the presence of ethidium bromide. The concentrations of p21 were 20 $\mu\text{g ml}^{-1}$ (lanes 3 and 7) and 40 $\mu\text{g ml}^{-1}$ (lanes 4 and 8). *b*, The autoradiogram was quantified by a Fuji Bio Imaging Analyzer. The repair (▨) and replication (■) signals in the absence of p21 (lanes 2 and 6 of *a*) are designated as 100%. The UV-specific repair synthesis was normalized against the background synthesis of the non-irradiated plasmid.

METHODS. RKO cell extract was prepared as described²⁶. The protein concentration of the extract was 5–10 mg ml⁻¹. The SV40 DNA replication and *in vitro* repair reactions were done as described^{9,27}. A typical replication and repair reaction contained 2 mg ml⁻¹ of extract protein. A repair reaction contained 10 µg ml⁻¹ each of the non-irradiated and UV-irradiated plasmids (at a UV dosage of 450 J m⁻²). The crude extract and p21 were mixed and preincubated on ice for 30 min; DNA templates were added and the reaction was further incubated for 2 h at either 30 °C (repair) or 37 °C (replication). Histidine-tagged p21 was expressed and purified as described⁷. The DNA templates were prepared by a Qiagen plasmid kit and sucrose gradient centrifugation⁹.

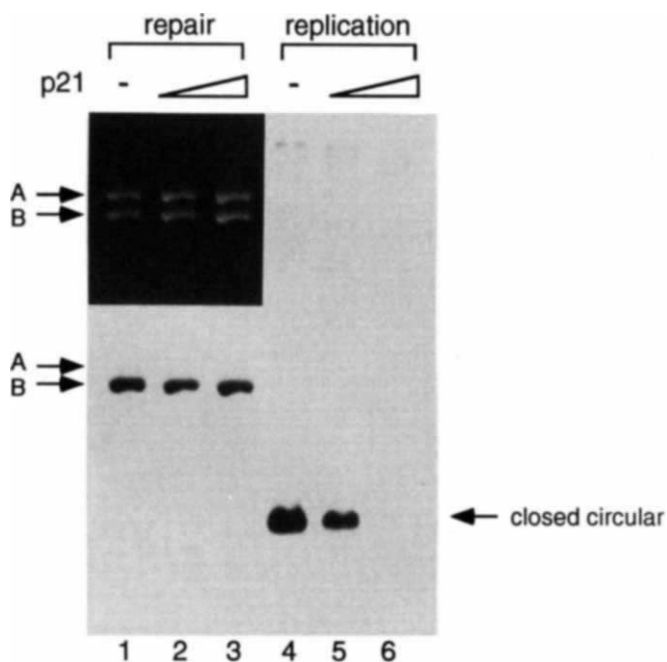


FIG. 2 Effects of p21 on DNA repair and DNA replication in a whole cell extract from a healthy human lymphoblast. *In vitro* repair (lanes 1–3) and SV40 DNA replication (lanes 4–6) were carried out essentially as described for Fig. 1. Also shown in lanes 1–3 is a photograph (top) of the same ethidium-bromide-stained gel. The two concentrations of p21 used in this experiment were $2 \mu\text{g ml}^{-1}$ (lanes 2 and 5) and $5 \mu\text{g ml}^{-1}$ (lanes 3 and 6). The human lymphoblast cell line GM02184 (purchased from NIGMS human genetic mutant cell repository at Camden, NJ) were grown under the conditions given by the supplier. Whole-cell extract was prepared following the protocol described previously²⁶.

stranded DNA-binding protein required for both SV40 DNA replication¹³ and nucleotide-excision repair¹⁴. We tested the effect of p21 on DNA repair using known amounts of both PCNA and p21 in this partially reconstituted system. The concentration of purified p21 (monomer) reached a 240-fold (Fig. 3, lane 7) or 60-fold (lane 8) molar excess over PCNA (trimer), but the *in vitro* repair was not significantly affected (Fig. 3: compare lanes 4 and 5 with 7 and 8). SV40 DNA replication requires much more PCNA than repair of ultraviolet-damaged DNA *in vitro*^{8,15}, yet repair is refractory to p21 whereas replication is p21-sensitive. Taken together, these data suggest that p21 can distinguish between the activities of PCNA in DNA replication and those in DNA repair.

FIG. 3 Characterization of the effect of p21 in a reconstituted PCNA-dependent repair assay. *In vitro* repair reactions were carried out in either the crude RKO cell extract (lane 1) or a fractionated extract (fraction II; lanes 2–8) in the presence or absence of 6 $\mu\text{g ml}^{-1}$ of RPA, and in the presence or absence of PCNA (0.1 $\mu\text{g ml}^{-1}$ for lanes 4 and 7 or 0.4 $\mu\text{g ml}^{-1}$ for lanes 5 and 8). The concentration of p21 used in lanes 6–8 was 5 $\mu\text{g ml}^{-1}$. Plasmids A (non-irradiated) and B (UV-irradiated) were the same as those used in Figs 1 and 2. The top panel shows a photograph of the ethidium-bromide-stained gel, and the lower panel represents an autoradiogram of the same gel.

METHODS. The whole-cell extract from RKO cells was fractionated on a phosphocellulose column as previously described²⁸, with some minor modifications. Essentially, the extract was adjusted to 0.2 M NaCl and applied to the column. The flow-through (fraction I) contained all the replication protein A (RPA) and most of the PCNA from the input extract. The high-salt eluate from 0.2 to 1 M NaCl (fraction II) contained all the other repair proteins. The residual small amount of PCNA retained in fraction II was further removed by an anti-PCNA monoclonal antibody (PC10) coupled to protein A–Sepharose beads. The resulting fraction was completely depleted of both RPA and PCNA as judged by ECL western blot detection (Amersham) and the *in vitro* repair assay. RPA used here was purified from human 293 cells²⁷.

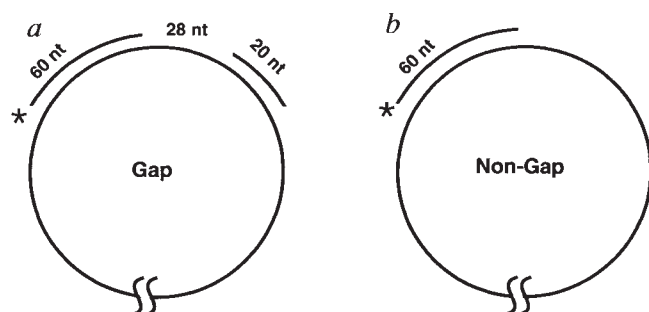
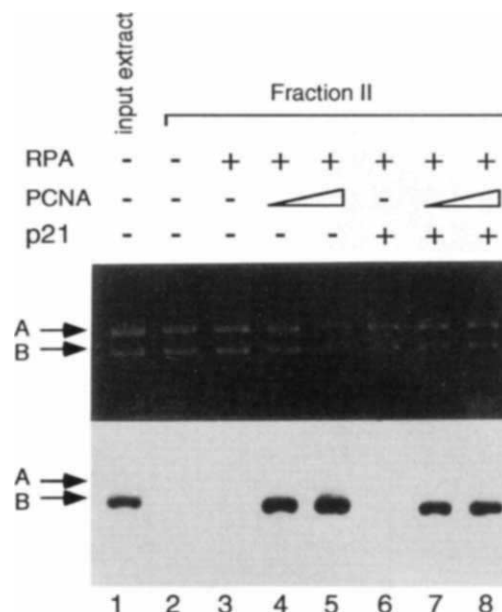
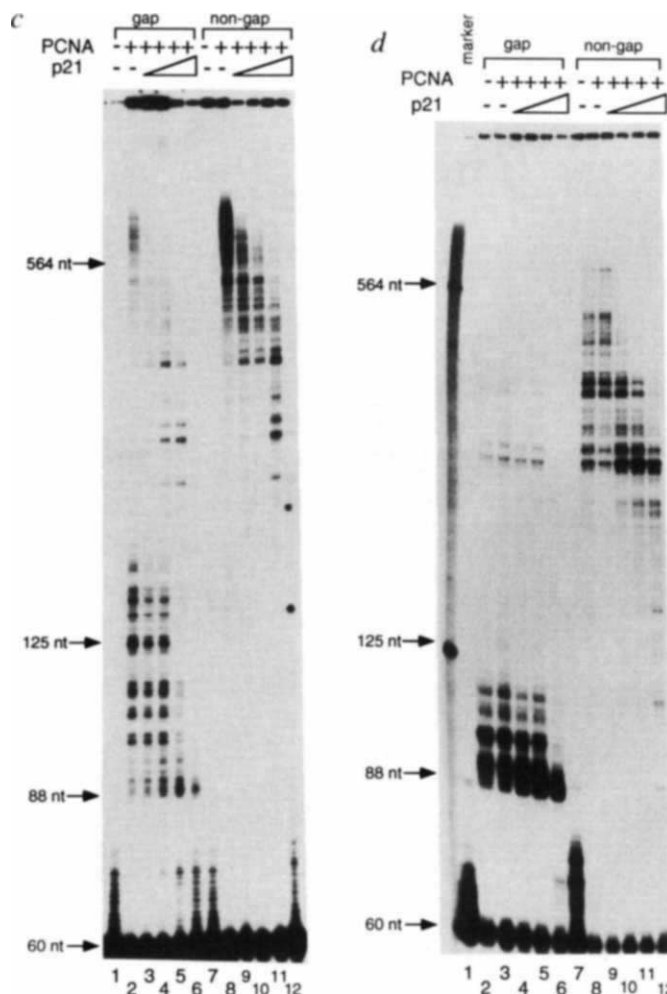


FIG. 4 p21 preferentially inhibits synthesis of long DNA fragments by polymerases δ and ϵ . *a, b*, Schematic diagrams of the DNA templates. The numbers indicate the lengths (in nucleotides) of the two primers and the single-stranded gap between them. The star represents the radioactive labelled end. *c*, The polymerase δ assay was carried out using either a gapped template (lanes 1–6) or a non-gapped template (lanes 7–12). The final concentrations of p21 used in lanes 3–6 and 9–12 were 2, 4, 8 and 16 $\mu\text{g ml}^{-1}$, respectively. The positions of different lengths of DNA fragments are indicated in nucleotides (nt). The position of 60 nt is the 3' end of the labelled primer. *d*, Same as *c*, but DNA synthesis by polymerase ϵ .

METHODS. A primer of nucleotides 928–987 in pBluescript SK(–) (Stratagene) was labelled and annealed to the single-stranded form of pBluescript SK(–) either alone (non-gapped) or with a primer of nucleotides 880–899 (gapped). The labelled and unlabelled primers were in 2- and 10-fold molar excess over the single-stranded DNA template, respectively. The polymerase assays were carried out in 40 mM Tris, pH 7.8, 1 mM dithiothreitol, 100 $\mu\text{g ml}^{-1}$ BSA, 7 mM MgCl_2 , 2 mM ATP, 120 μM dNTP, 4 $\mu\text{g ml}^{-1}$ DNA template, 40 $\mu\text{g ml}^{-1}$ RPA, 36 $\mu\text{g ml}^{-1}$ replication factor C (RFC; fraction IV), and when indicated, 4 $\mu\text{g ml}^{-1}$ PCNA, 1 unit of polymerase ϵ and 0.1 units polymerase δ per 10- μl reaction (for unit definition, see refs 27, 29). For PCNA-dependent synthesis by polymerase ϵ , NaCl was added to a final concentration of 125 mM. The amounts of the two polymerases were chosen to generate approximately an equivalent amount of DNA synthesis on the primed-ssDNA templates. Calf polymerase δ and human RFC were purified as described²⁷. Calf polymerase ϵ was a gift from U. Hubscher at University of Zurich–Irchel, Switzerland²⁹. p21 was preincubated on ice for 30 min with the rest of the reaction mixture minus the DNA substrate. DNA substrate was added and the reaction was further incubated at 37 °C for 1 h. The reaction products were analysed on a 6% denaturing acrylamide gel.



Nucleotide-excision repair in mammalian cells can be separated into two steps¹⁶. First, the damaged DNA site is recognized and a fragment of 27–29 nucleotides containing the pyrimidine dimer is released by an incision/excision step, then PCNA-dependent repair synthesis closes the gap. In contrast, replicative DNA synthesis extends over much longer regions of DNA. It seemed plausible that the structural differences in DNA templates could lead to the differential effects of p21. Alternatively, a PCNA-dependent DNA polymerase other than polymerase δ , such as polymerase ϵ , could be responsible for repair synthesis and be more resistant to p21 inhibition. For example, polymerase ϵ requires less PCNA than polymerase δ for polymerase activity¹⁷.

To test these models, we designed two DNA templates mimicking those involved in either DNA repair or replication. The first template contains two oligonucleotides annealed to a circular, single-stranded DNA, creating a 28-nucleotide gap (Fig. 4a). In this case, the upstream primer is labelled and the downstream primer serves as a 'terminator' that will retard progress of the DNA polymerase. The second template is a primed single-stranded DNA without the second oligonucleotide, thus resembling a template for DNA replication (Fig. 4b). Synthesis by polymerase δ or ϵ was carried out in the presence of replication protein A, replication factor C (a DNA-polymerase accessory factor) and PCNA (where indicated).

As shown previously^{17–20}, polymerase δ activity was dependent on the presence of PCNA (Fig. 4c, compare lanes 1 and 7 with 2 and 8). In the case of the gapped template, the polymerase paused at multiple sites near the 3' end of the gap sequence (88 nucleotides); in addition, there was a considerable amount of strand displacement synthesis beyond the gap. Synthesis of long DNA products (for example, above the 564-nucleotide marker) on both templates was greatly diminished by increasing amounts of p21 (Fig. 4c, lanes 3–6, 9–12; the molar ratio of p21 to PCNA in lanes 6 and 12 was 20 to 1). Alkaline agarose gel electrophoresis confirmed that synthesis of larger DNA products (500–2,000 nucleotides) was greatly reduced by p21 (data not shown). In contrast, PCNA-dependent gap-filling DNA synthesis was relatively unaffected (Fig. 4c, lanes 3–6; 88 nucleotides). The effect of p21 on polymerase ϵ activity was assessed using conditions under which the polymerase activity was PCNA-dependent (Fig. 4d). Again, p21 blocked synthesis of long DNA fragments (Fig. 4d, lanes 9–12; around the 564-nucleotide marker), but not the short patch synthesis by polymerase ϵ (Fig. 4d, lanes 3–6; 88 nucleotides). Compared with polymerase δ , synthesis by polymerase ϵ on the gapped templates was more confined to the gap region (Fig. 4d, lanes 1–6), and its PCNA-dependent activity seemed to be more resistant to p21, suggesting that it was a better polymerase for DNA repair synthesis (for example, compare lanes 8 and 9 in Fig. 4c with those in Fig. 4d). Notably, both polymerases have been implicated in DNA repair^{21–23}. Although our results do not address which polymerase plays a major role in nucleotide-excision repair *in vivo*, they show that short-gap synthesis by either polymerase is relatively insensitive to inhibition by p21.

PCNA has a dual function in SV40 DNA synthesis¹³. It recognizes a primer–template junction in cooperation with replication factor C and facilitates loading of polymerase δ ; PCNA also enhances the processivity of polymerase δ during the elongation step. We suggest that the role of PCNA in processivity may be more sensitive to p21 than is its role in polymerase loading, and the PCNA–p21 interaction may arrest the polymerase and cause it to fall off the template. In repair synthesis, it is conceivable that the primary role of PCNA is to help the polymerase (δ or ϵ) localize to the junction of the incised DNA. Once bound to the template, the polymerase may be able to synthesize the short patch of DNA without the aid of PCNA. In addition, other repair-specific proteins^{24,25} may further contribute to immunizing the repair machinery to p21.

DNA damage poses a tremendous challenge to the integrity of the genome. The post-damage events in normal cells ensure that any errors caused by the damage are corrected before propagation of the chromosomes. Based on our current understanding, elevated p21 levels block cell-cycle progression and DNA replication by coordinately inhibiting the functions of both CDKs and PCNA. The results presented here suggest a selectivity in p21 function which allows it to arrest DNA replication while permitting active DNA repair. p21 and its interaction with CDK, cyclin and PCNA may also be involved in normal proliferating cells. It is possible that p21 interaction with PCNA couples DNA repair to DNA replication during S phase and if the link between p21 and PCNA is disrupted, post-replicative repair could be abrogated, leading to genetic damage. We suggest that p21, PCNA and its partner, replication factor C might function, perhaps in cooperation with cyclin and CDK, to coordinate the replication and repair machineries with cell-cycle progression in both normal and damaged cells. □

Received 13 July; accepted 18 August 1994.

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ACKNOWLEDGEMENTS. We thank U. Hubscher for purified calf thymus DNA polymerase ϵ , M. Kastan for the RKO cell line, and S. Bell, W. Herr, P. Kaufman, T. Melendy and A. Stenlund for critical reading of the manuscript. This research was supported by a NIH grant to B.S. R.L. is a special fellow of the Leukemia Society of America. G.H. is a fellow of the Damon Runyon-Walter Winchell Cancer Research Fund. D.B. is an Investigator of the Howard Hughes Medical Institute.

ERRATUM

Mice with targeted disruptions in the paralogous genes *hoxa-3* and *hoxd-3* reveal synergistic interactions

Brian G. Condle & Mario R. Capecchi

Nature **370**, 304–307 (1994)

FIGURES 1 and 2 in this Letter were accidentally transposed during the production process. The legends are correct as published. □

One in the eye

A double insight into visual accommodation by the eye lens in birds and into the activity of a superfamily of metabolic enzymes is provided by the structure of turkey lens δ -crystallin.

THE lens of the eye presents an interesting biomechanical puzzle: not only must it be transparent to allow the passage of light, and supply a refractive index gradient to avoid aberration, but its constituent materials must be highly stable to insult by ultraviolet light, radicals and so on because lens proteins cannot be regenerated. One might imagine that such demanding requirements would have led to the evolution of a highly specialized lens material. In reality these criteria are met by a motley collection of proteins — mainly metabolic enzymes or their relatives that have been 'hijacked' into the lens during evolution. The structure of one of these constituent proteins, δ -crystallin, is reported in this month's *Nature Structural Biology*¹ and, as well as describing another new protein fold, it sheds light on both the protein's role as a crystallin and its ancient function as an enzyme of the urea cycle.

The α - and β/γ -crystallins are ubiquitous in vertebrates, indicating that these proteins were recruited to the eye at the very beginning of lens evolution. The α -crystallins are similar to the small heat-shock family of proteins and the β/γ -crystallins share homology with the spore coat protein S from *Myxococcus xanthus*.

δ -Crystallin, on the other hand, is a taxon-specific crystallin found only in birds and reptiles, suggesting that it was recruited to the lens in a common ancestor of all reptiles and birds after divergence from the line leading to mammals^{2,3}. All the taxon-specific crystallins are either fully paid-up enzymes or are closely related to enzymes: δ -crystallin shares over 90% sequence identity to argininosuccinate lyase (ASL), an enzyme involved in arginine biosynthesis and, in ureotelic species, the urea cycle. ASL and δ -crystallin (which no longer has enzymic activity) form part of a superfamily of metabolic enzymes, including fumarase, aspartase and adenylosuccinase, so the

new crystallin structure will provide a template for the structures of its more distant relatives.

Like all the other members of the superfamily, δ -crystallin is a tetramer. The most striking feature of the overall structure is its highly helical nature. Each of the subunits of the tetramer consists of three domains. Domains 1 and 3 have a similar topology: two helix-turn-helix motifs stacked at right angles to one another, which may have arisen by partial gene duplication. These two domains are joined through domain 2, which consists for the most part of a five-helix bundle. Two of these subunits pack tightly together in a head-to-tail fashion and the tetramer is formed from a dimer of these dimers. The subunit contacts are mainly between the helix-bundle domains, and for part of their length they form an impressive 20-helix bundle — the compact heart of the tetramer.

The lens is one of the very few tissues in vertebrates where the molecules that were laid down during embryonic development persist throughout adult life. The lens cells, as they develop, synthesize large amounts of protein, of which over 90% are crystallins. Nuclei and other organelles are then lost and the cells elongate, reaching lengths of up to a centimetre, and migrate away from the front of the lens. The lack of protein synthesis in the differentiated fibre cells means that, to maintain the transparency and refractive properties of the lens, the crystallins must remain intact for the entire lifetime of the organism. The symmetrical nature of the heart of the δ -crystallin tetramer, and of the repeated Greek key motifs of the β/γ -crystallins, is thought to be important in stabilizing proteins in the lens.

The recruitment of such a large α -helical protein to the lens of the avian eye probably serves a second function. The amount of water in the lens correlates with its refractive power and softness, and hence its ability to accommodate. The wide-ranging visual accommodation peculiar to the eyesight of birds requires the centre of the lens to have liquid-like properties. In the crystals, δ -crystallin interacts mainly with the solvent and makes few contacts with other tetramers. On the other hand, the lens in most other vertebrates (the short-sighted rat, for example) has a glass-like centre, consisting of the small β - and γ -crystallins, and in these

crystals protein-protein interactions are much more extensive.

The crystallins also offered a new perspective on molecular evolution when it was discovered that the genes for ϵ - and τ -crystallins also encode lactate dehydrogenase-B, and α -enolase, respectively. This phenomenon of gene 'sharing'^{2,3} — whereby a protein acquires a new function apparently unrelated to the original — is thought to have arisen through modification of gene expression, and could explain how the ancestral ASL gene gave rise to δ -crystallin. In the chicken, both genes have a lens-preferred enhancer, suggesting that ASL was recruited to the eye before gene duplication took place. Presumably the constraints of the new lens-specific function of ASL were not consistent with optimal enzyme activity; these two independent evolutionary pressures then favoured gene duplication and further independent selection.

Nonetheless, the high degree of similarity between δ -crystallin and ASL allow Slingsby and colleagues¹ to make deductions about the catalytic mechanism of the latter and indeed about the superfamily as a whole. The most highly conserved regions of the proteins of the superfamily cluster in the same region to form a cleft, around which are a bevy of almost invariant residues that are for the most part well placed to affect catalysis.

The variety of proteins found in the lens would suggest that recruitment is a fairly neutral process, but it is hard to believe that the crystallins are selected purely at random. Most of the crystallins are related or identical to oxoreductases that bind pyridine nucleotide cofactors, which may act as ultraviolet filters and reduce glare. A connection has also been noted with enzymes associated with carbohydrate metabolism, many of which are induced by heat shock, suggesting that the crystallins play a role in stress tolerance⁴. It will be interesting to see whether the crystallins have functions apart from acting as packing material in the lens.

Guy Riddihough

Guy Riddihough is Editor of Nature Structural Biology.

Also in this month's *Nature Structural Biology*: Max Perutz celebrates the life and achievements of Linus Pauling; catching enzymes in the act — inosine bound to adenosine deaminase and the NADPH-trichosanthin complex; parallel evolution of the control of yeast phosphorylase; heptad repeats and four-helix bundles; pectate lyase in complex with calcium; predicting new serine proteinase inhibitors; and designing receptor antagonists.

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New lines in labware

For the lab — a nitric oxide analyser, a ratio imaging microscope system for the life sciences, a device for producing digital images from slides, hyphenated systems and a selection of detectors.

SIEVERS Instruments has developed a system to determine nitrate and nitrite concentration in biological fluids such as serum, cell cultures and tissue perfusate using the Model 270B **nitric oxide analyser** (*Reader Service No. 100*). Based on reduction with vanadium chloride in hydrochloric acid, the system consists of a purge vessel with a heating water jacket and cold-water condenser, and a gas bubbler trap for the HCl vapours. Nitrate and nitrite are converted to nitric oxide and detected by ozone-induced chemiluminescence. The detection limit is said to be about 1 pmol of NO_3^- , the analysis time is about a minute per sample, and the sample size can range from a few microlitres to several millilitres.

Varian's new Gemini 2000 **NMR spectrometer** has been combined with the computing clout of a Sun Microsystems SPARCstation (*Reader Service No. 101*). It is designed to perform fully automatic experiments such as COSY, DEPT and HETCOR, and to deliver ^1H spectra in 4 min or less. Design capabilities of the Gemini 2000 include indirect detection, pulsed-field gradients, solvent suppression and spin locking. To meet the needs of any user, both a simplified and an advanced software interface are provided. The GLIDE interface is intended to make the Gemini 2000 simple to operate, with drop-down menus, online help and fully adjustable parameters. For more advanced capabilities, the VNMR interface provides data analysis tools such as linear prediction, digital signal processing, deconvolution and interactive FID weighting. Various probes are available, including the pulsed-field gradient indirect detection probe for proton-carbon and other heteronuclear

correlation experiments, the Nano•nmr probe and the Auto•nmr probe for high-throughput laboratories. Gemini 2000 spectrometers are available with Oxford superconducting magnets from 200 to 400 MHz.

A new sanitary, benchtop **tangential filtration system**, called the ProFlux M12, has been developed by Amicon (*Reader Service No. 102*). The system is intended for the concentration or purification of solutions from laboratory size to small-scale production of up to 100 litres. Suggested applications include aseptic processing, concentration of biological solutes, cell harvesting and washing, the removal of bacteria and pyrogens and the concentration of plasma factors. The system is available with a choice



ProFlux M12 tangential filtration system: offers quick cartridge changeovers.

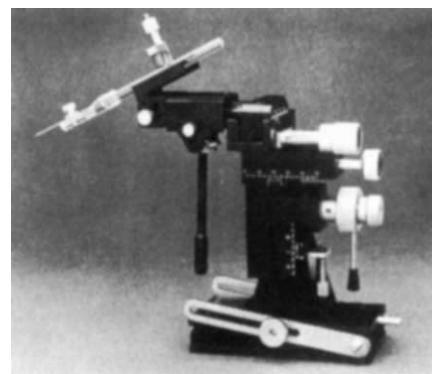
of cartridges for tangential ultra- and microfiltration with spiral, hollow-fibre or cassette-type filtration. Tubing, fittings and the reservoir can be autoclaved.

■ Microscopy/image analysis

Attofluor Ratiovision from Carl Zeiss is a **ratio imaging microscope system** with applications in cell biology, pharmacology, medicine and physiology (*Reader Service No. 103*). What is analysed is the mode of action of free ions and messengers in the cellular signal path when biological processes such as muscle contraction, nerve impulse transfer, hormone release, cell division and immunological response, are activated. The system is used for the TV-based, image analytical two-dimensional display and the quantification of the concentration of these intracellular ions (Ca^{2+} , H^+ , Mg^{2+}) and second-messenger molecules (for example, cAMP). Fluorescence dyes such as Fura-2, BCECF, Indo-1, Fluo-3, and energy-transfer dyes are used as evidencing fluorochromes. Atto Instruments produces

the ratio-imaging components for Zeiss and has tailored them to the Axioskop, Axioplan, Axiophot and Axiovert microscopes from Zeiss with their special ICS Fluor objectives.

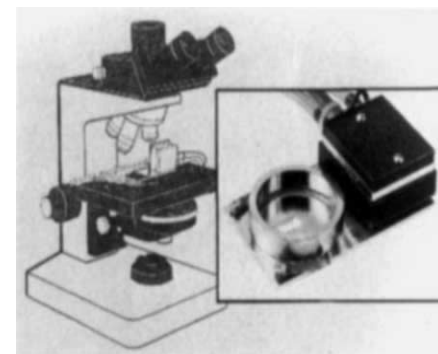
The new MMJ **manual micromanipulator** from World Precision Instruments is designed to simplify oocyte injection and other tasks requiring repeated raising and lowering of a tooltip (*Reader Service No. 104*). Fine adjustment is under the control of



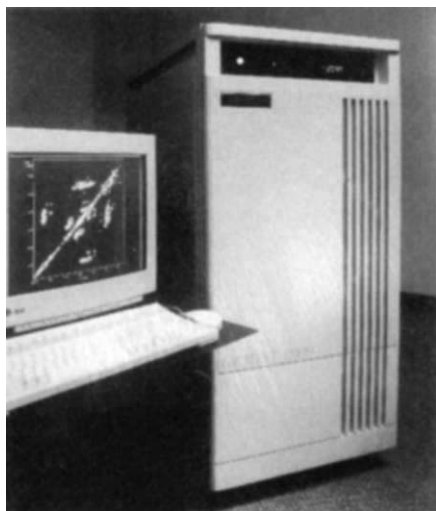
Joystick-controlled micromanipulator.

a single wand, which is said to pivot 360° for x - y positioning, and twirls clockwise-counterclockwise for vertical placement. A separate coarse adjustment lever is designed to raise the tooltip out of the focusing plane without disturbing the fine settings, then to return it to its previous position.

Custom **thermal stages** are now available for Physitemp's TS-4 and TS-4ER (*Reader Service No. 105*). These controllers maintain stage and specimen temperature to between -20°C and 100°C . The custom stages are made to the user's specifications and can be a simple modification of the standard TS-4 stage or a completely new configuration. Both standard and inverted microscopes can be accommodated. The



Custom-made thermal stages.



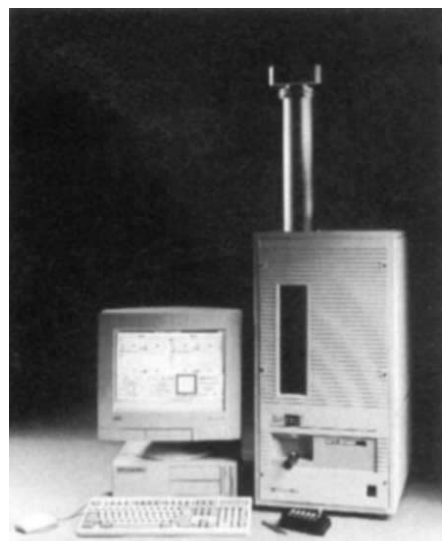
Gemini 2000 NMR spectrometer.

standard stage is designed for use with glass slides. Custom versions are now made for many other applications: collars for tissue culture dishes, miniature formats, flat plates without centre holes. The TS-4 thermoelectric device needs 110 or 220 V a.c. and a trickle of running water for continuous operation. Temperature control is said to be accurate to $\pm 0.1^\circ\text{C}$.

SprintScan 35 is the new 35-mm slide scanner from Polaroid that can be used to convert high-quality images into digital form in under 30 s (*Reader Service No. 106*). Priced at under UK£2,500, the SprintScan 35 automatically corrects colour and sharpens the image during the scanning process. Images are scanned in a single pass and are output at resolutions of up to 2,700 dots per inch. SprintScan accepts any colour or black and white 35-mm transmissive film media, including positive and negative, mounted or unmounted transparencies and film strips. It is compatible via a standard SCSI-2 interface with any Apple Macintosh or Windows-based personal computer. For Mac users, the scanner is shipped with a plug-in for Adobe Photoshop; for the Windows environment, scanner software includes a Twain driver and a Twain-compatible scanning utility.

■ Mass spectrometry

The new BioPro Ez1000 matrix-assisted laser desorption **time-of-flight mass spectrometer** (MALDI-TOF/MS) from Biosearch, a subsidiary of Millipore, has been specifically designed with biochemists and technicians in mind (*Reader Service No. 107*). It offers users a means for the rapid analysis and confirmation of peptides, proteins and oligonucleotides. Stated system specifications include an accuracy of up to 0.01 per cent, resolution in excess of 1,300, a sensitivity in the sub-picomole range and a mass range to greater than 185,000 daltons. The system's graphical user interface guides the user through the steps of acquiring data



Time-of-flight mass analysis system.

and then analysing the results. SmartShot data acquisition software is provided. EzPrep sample analysis substrates, available in different configurations, are designed to simplify sample preparation for MALDI-TOF/MS.

Hewlett-Packard has launched a new atmospheric pressure ionization **electrospray liquid chromatograph/mass spectrometer** (API-electrospray LC/MS) system (*Reader Service No. 108*). The system can be coupled with conventional HPLC methods and automatically matches masses to amino acid sequences generated by protein digests. It allows the characterization of peptide maps at picomole levels using on-line LC/MS. The molecular weights of peptides, intact proteins and oligosaccharides up to 150,000 daltons can be measured. A high-flow sprayer is intended to provide the required sensitivity and reproducibility at flow rates from 1 to 1,000 $\mu\text{l min}^{-1}$ and with a range of mobile phases and mobile-phase additives. Accompanying Microsoft Windows-based software automates routine instrument control and data interpretation tasks.

■ Detectors

The new MD 800 **liquid chromatography detector** (LCD), developed by Fisons Instruments/Mass Detector Systems, is designed for the positive identification of unknown peaks in a single chromatographic run in a way that provides increased throughput and reduces the time taken for methods



MD 800: liquid chromatography detector.

development (*Reader Service No. 109*). Users have the option of acquisition with library searchable structural information, or molecular weight confirming chemical ionization detection, which comes into its own when working with novel synthetic compounds not found in libraries. Detection limits are said to be in the parts-per-billion range and can be further increased by using the negative chemical ionization mode. Both EI and CI techniques are available on the LCD. The LCD is controlled by the MassLab data system which operates in the Microsoft Windows environment. Mass detector acquisition parameters can be defined and saved. During the acquisition process, MassLab calculates final concentrations and allows the user to customize the report format.

ATI Unicam announces the introduction of the Crystal 1000 **conductivity detector** for capillary ion electrophoresis (*Reader Service No. 110*). This 'end capillary' detection system, which measures direct conductivity, is intended for the analysis of both



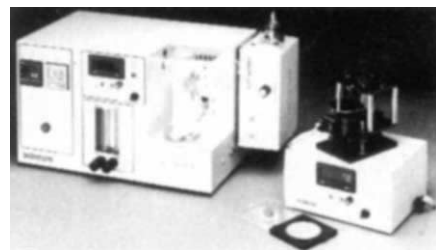
ATI Unicam's Crystal 1000: measures direct conductivity.

ionic and organic anions and cations. It has been designed to overcome some of the disadvantages seen with indirect ultraviolet detection methods. The cell design has an effective volume of less than 3 nl. The detector is intended for use with the company's Crystal 300 Series modular capillary electrophoresis injector system.

Perkin-Elmer's new Series 200 **refractive index detector** is a liquid chromatography and gel permeation chromatography detector intended for use with compounds that do not have high absorptivity in the ultraviolet range, such as polymers, sugars, organic acids and triglycerides (*Reader Service No. 111*). The detector, which is of a deflection-type design, features internal temperature control of the flow cell, offset adjustment, autozero and autopurge of the reference cell. The last two features make unattended, routine analyses or methods development easy, says the company, because they enable automatic solvent changes and baseline adjustments. The Series 200 RI detector provides detection with a variety of mobile phases with its broad refractive index range (1.00–1.75 RIU) without the need to change prisms.

■ Chromatography

The LC-Transform, distributed by Viscotek, is designed to overcome the problems of solvent adsorption or laborious solvent elimination by preparative chromatography typically associated with interfacing the identifying power of FT-IR with the resolving



Chromatography/FT-IR interface.

ability of a range of separation techniques, including HPLC, GPC and field-flow fractionation (*Reader Service No. 112*). With this instrument, the effluent from a chromatograph is directed to a gas-flow nozzle which sprays the fluid onto the perimeter of a thin Ge disk; during this process the mobile phase is evaporated, leaving a track of dry solvent-free spots of individual solutes for FT-IR analysis. The Ge disk is then loaded onto an optical unit, which slots into the sample compartment of any FT-IR spectrometer. Under rotational control, the deposited chromatogram is brought to the FT-IR focus of the optical module for sequential analyses. Utilizing an off-line configuration, the LC-Transform frees both the chromatograph and the FT-IR instrument for other uses. Intended uses include product deformation, compositional polymer analysis, identification of drug intermediates and profiling trace components.

Three solvent gradients, flow rates of 0.01–10 ml min⁻¹ and variable ultraviolet detection are some of the features of the new **HPLC system** — the TSK HPLC System-10 — now available from NOVEX (*Reader Service No. 113*). The system's control module provides a means of entering simple



Novex's compact HPLC system.

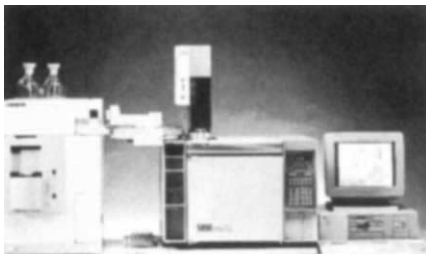
or complex gradient profiles and stores up to eight methods. Available options include computer control and data collection, a refrigerated variable-volume autosampler, nonmetallic flow path, UV/visible detection and a gradient monitor to measure the solvent profile.

Oxford GlycoSystems introduces new **columns** for the two-dimensional profiling of complex oligosaccharide mixtures and fractionation of discrete sialylated and neutral glycans (*Reader Service No. 114*). The GlycoSep HPLC column set consists of two columns: GlycoSep C, an anion-exchange column for profiling and fractionating oligosaccharide mixtures on the basis of their overall negative charge, and the GlycoSep H, which is intended for profiling and fractionating mixtures of the basis of their hydrophobicity. Chromatographic conditions have been optimized specifically for

the separation of oligosaccharides labelled with the fluorescent dye 2-aminobenzamide using the company's new Signal Labelling Kit. The column set contains both columns together with standards and application protocols. Detection wavelengths are excitation 330 nm, emission 420 nm.

■ Supercritical fluid extraction

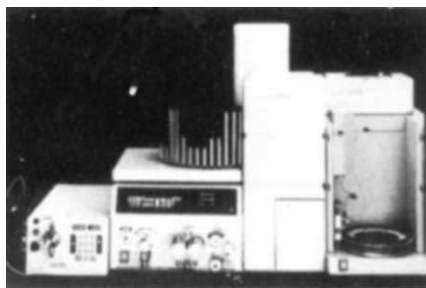
Last month, Hewlett-Packard introduced an optional software and hardware **bridge that links supercritical fluid extraction with chromatographic analysis** (*Reader Service No. 115*). The bridge, which integrates the HP 7680T supercritical fluid extractor



Hewlett-Packard's SFE bridge.

(SFE) with an HP chromatograph, enables unattended automation of sample preparation and analysis, from sample thimble insertion into the HP SFE through chromatographic analysis and generation of the final analytical report. It can be integrated with an HP 5890 Series II gas chromatograph and HP 5971 or 5972 mass spectrometer, or with the HP 1050 series high-performance liquid chromatograph for total automation. The HP 7680T SFE bridge software provides control of the analytical system and operates within a Microsoft Windows environment.

Varian now offers two **supercritical fluid extraction systems** for preparing samples before chromatographic analysis, which are designed to save time, improve accuracy and eliminate the use of toxic solvents (*Reader Service No. 116*). The AutoPrep 44



See Varian for a choice of supercritical fluid extractor systems.

is a fully automated, standalone system that can be programmed automatically to perform up to 44 sequential extractions; the PrepMaster, on the other hand, is operated manually. Both systems are intended for supercritical fluid extraction of pollutants, pesticides, flavours, fats, drugs of abuse, food additives and other substances. A continuous flow CO₂ pump on both

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models eliminates the need for refilling between extractions.

■ **Miscellaneous devices/gadgets**
Lake Shore's model 410 **hand-held gaussmeter** is designed for magnetic field measurements from 0.1 G to 20 kG (0.01 mT to 2 T) (Reader Service No. 117). The 410 displays in gauss or tesla, a.c. or d.c. magnetic field values with a stated 100-mG resolution on the 200-G range. The memory

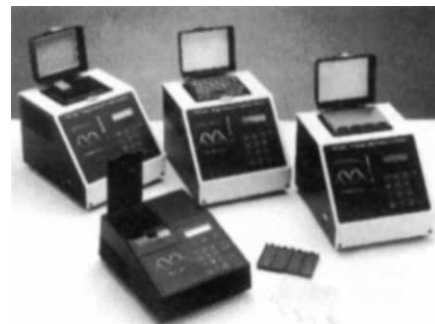


Hand-held gaussmeter from Lake Shore.

hold function stores in nonvolatile memory the complete configuration of the instrument, including the calibration number and the probe offset, making it unnecessary, in most cases, to go through a setup procedure when powering up the instrument. The model 410 provides a zero probe function used to eliminate probe offsets and small external fields and a filter function is used when there is 'noise' in the field being measured.

Need to **measure the zeta potential** of a particle? Galai has introduced the Zeta Potential Accessory (ZPA) which has been designed to be easy to operate and clean and for which no calibration is required (Reader Service No. 118). The dynamic shape characterization channel lets the user view the particles as they are analysed. Because it is based on a modular system, the ZPA offers particle information in addition to zeta potential, namely particle size distribution, particle shape distribution and particle mobility distribution. The ZPA measurement is based on an analysis of particles using the conventional electrophoresis procedure. The special ZPA cell is set between the video microscope and the synchronized strobe illumination source in a Galai Particle Shape and Size Analysis Instrument: either the DSA-10 with its special open architecture, or the fast CIS-100 with the additional laser measurement channel.

New **sample block and heated lid accessories** are newly available for use with GRI's PTC-100 thermal cycler range (Reader Service No. 119). Made from silver, the sample block is designed to enable researchers to process multiple samples in 96-well, V-bottomed microplates, with faster ramping



GRI's thermal cycler accessories.

speeds than standard blocks. In addition to microplates, the block accommodates 0.2-ml V-strip and individual tubes for normal and rapid single reaction applications. The Hot Bonnet heated lid accessory eliminates the need for oil overlays. By creating a temperature differential in the reaction tube, the formation of condensation is said to be prevented. The lid has its own power supply and fits over both microfuge tubes and microplates.

Mettler Toledo's AE range of balances has now been superseded by the AG **analytical balances** (Reader Service No. 120). The AG series are said to take up 30 per cent less bench space and feature a higher draft shield, which allows weighing into relatively large vessels such as volumetric flasks. The weight range of the AG balances is the same as that of the AE series. A choice of four models is offered with a weighing range of 0-210 g; the AG245 has an additional weighing range of 0-41 g, with a stated readability of 0.01 mg. Design features include the LocalCAN universal interface built-in as standard, selectable units, a formula weighing programme, dynamic weighing and piece counting. All are equipped with an internal weight which can be used for calibration to full load at a keystroke. The user may also calibrate the balance with selectable external weights or check it with the internal or an external weight. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details on any of the products featured, fill in the reader service card bound inside the journal.

CORRECTION

The SequiTherm Cycle Sequencing kit, as evaluated in 8 September product review (Nature 371, 182; 1994), is a registered trademark of Epicentre Technologies, USA and distributed by Cambio Ltd in the UK.